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ENZYMATIC DETERMINATION OF ADENOSINE TRIPHOSPHATE USING YEAST HEXOKINASE*

By FUMIO IMAMOTO, KOZO IWASA and KAZUO OKUNUKI

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(Received for publication, March 20, 1957)

Although it is customary to measure the amount of acid-labile phosphorus, the so-called Δ7P, in order to determine the content of adenosine triphosphate (ATP) in physiological materials, some enzymatic methods for that were studied by several laboratories. Kornberg (*1*) reported a method for determination of ATP using hexokinase and glucose-6-phosphate (G6P) dehydrogenase by measuring the increase of the reduced triphosphopyridine nucleotide. Kalckar (*2*) determined ATP by measuring the deamination of adenylic acid spectrophotometrically, using adenyl pyrophosphatase, adenylate kinase, and adenylic acid deaminase. Some modifications of the method of the deamination were described by Albaum and Lipshitz (*3*) and by Mackler, Foris, and Guest (*4*). Multienzyme systems were also applied to ATP assay; Slater (*5*) used the muscle glycolytic enzymes, and Strehler and Totter (*6*) used the luminous enzyme system from firefly. One enzyme reaction, *i.e.*, myosin ATP-ase was used by Bailey (*7*), Rowles and Stochen (*8*), Steyn-Parvé and Gerritsen (*9*), and by Bowen and Kerwin (*10*), and phosphoglyceric acid kinase by Holloway (*11*).

The present paper reports the results of experiments designed to determine ATP by measuring the diminution of the initial acid-labile phosphorus in the yeast hexokinase reaction. As a result of this investigation, it was concluded that this method is easy to use and is as sensitive as the other enzymatic determinations of ATP.

MATERIALS

ATP was prepared as barium salt from rabbit skeletal muscles according to the method of Du Bois, Albaum, and Potter (*12*). After purification using anion exchange resin (Dowex-1, X8) according to the method of Cohn and Carter (*13*), crystalline ATP was obtained by the method of Berger (*14*).

Adenosine diphosphate (ADP) was prepared from ATP by using lobster tail muscles (*15*).

Hexokinase was prepared by a modified method of Berger, Slein, Colowick, and Cori (*16*). The dried baker's yeast was suspended in three volumes of $M/20$ Na_2HPO_4 solution, incubated with mechanical stirring at 35° for 2.5~3.0 hours, and filtered through

* A preliminary report of this work was presented at the 27th Meeting of the Japanese Biochemical Society held in Kyoto, in April, 1955.

a layer of Hyflo-Super Cel. The crude enzyme was precipitated from the extract (Lebedew juice) with ammonium sulfate between 0.55~0.75 saturation, and the precipitation was repeated once more with ammonium sulfate. The crude hexokinase precipitate was stored at 0° in a refrigerator until use. The activity remained unchanged for more than three months. The enzyme showed the activity of 450 units according to Berger *et al.* (*16*), on manometric measurement of CO_2-evolution per minute per mg. of protein nitrogen. A more purified hexokinase was obtained when the crude enzyme solution was incubated with 2 per cent glucose at 60° for 2~3 minutes and the soluble portion was reprecipitated with a saturated solution of ammonium sulfate between 0.66~0.75 saturation. In this preparation, adenylate kinase, and phosphatases activity were never detected.

Racker-Slater enzymes were prepared according to Racker (*17*) and Slater (*10*).

Reduced diphosphopyridine nucleotide (DPNH) was prepared by hydrosulfite reduction of diphosphopyridine nucleotide (DPN), which was prepared from baker's yeast (*18*).

G6P was synthesized chemically according to the method of Seegmiller and Horecker (*19*) and was purified with anion exchange resin (Duolite A-40).

EXPERIMENTALS AND RESULTS

When the reaction mixture contains magnesium ions and an excess of glucose and yeast hexokinase, the enzyme reaction proceeds at a rate proportional to the amount of ATP. The difference between the amounts of the initial and residual acid-labile phosphorus indicates the amount of phosphorus transferred to glucose by hexokinase and such phosphorus is designated as "Incorporated phosphorus (Inco.-P)" throughout this report.

Three reaction systems for the estimation of Inco.-P are as follows:

	A (ml.)	B (ml.)	C (ml.)
M/5 Tris*-buffer pH 7.5	0.3	0.3	0.3
M/5 Glucose	0.1	0.1	0.1
M/80 $MgCl_2$	0.1	0.1	0.1
Hexokinase	0.3	0.3	nil
Unknown solution	x	nil	x
Water	1.0–x	1.0	1.3–x

After thorough mixing, each system is incubated at 30° for 15 minutes. Then the reaction is stopped by deproteinization with 0.2 ml. of 2 *N* $HClO_4$, and the mixture is centrifuged. The acid-labile phosphorus in the supernatant is determined in each reaction system according to the method of Fiske and Subbarow (*20*), and the Inco.-P is calculated from the following equation:

$$\text{Inco.-P}=(P_{10}^{C}-P_{0}^{C})-\{(P_{10}^{A}-P_{0}^{A})-(P_{10}^{B}-P_{0}^{B})\}-\{P_{0}^{A}-(P_{0}^{B}+P_{0}^{C})\}-P_{10}^{C}-P_{10}^{A}+P_{10}^{B}$$

Where P_{10} and P_0 in three systems indicate the amount of acid-labile phosphorus with and without heating in *N* H_2SO_4 at 100° for 10 minutes, respectively. The amount of ATP in the test solution should lie preferably in the range from 0.2 to 2.0 micromoles. The value of Inco.-P is equal to the amount of ATP, when they are expressed in moles, because the equillibrium of

* Trishydroxyaminomethane.

hexokinase reaction is extremely shifted to G6P. There is some possibility that the crude hexokinase preparation may be contaminated with adenylate kinase (*21, 22*) or ATP-ase (*23*). When concentration of the crude hekokinase was varied successively, the values of Inco.-P increased slowly beyond one-half of the total acid-labile phosphorus of ATP, and, in a certain preparation, a distinct two-steps curve was occasionally found (Fig. 1). It is suggested that a small amount of adenylate kinase is present in the preparation and in a diluted state, it does not affect the values of Inco.-P by hexokinase, probably owing to the dilution effect of adenylate kinase. In the case of highly

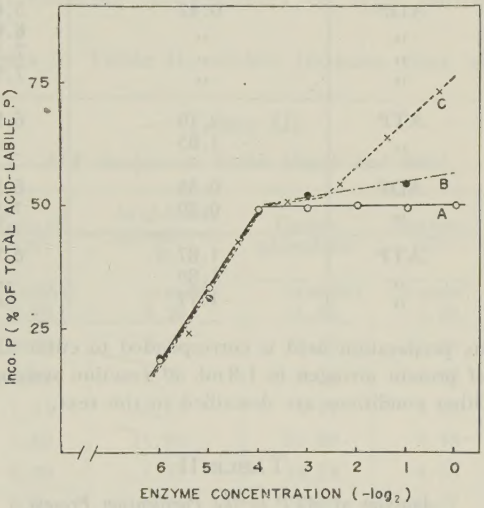

FIG. 1. Effect of enzyme concentration on Inco.-P. Conditions are as described in the text. Abscissa is the Inco.-P expressed in percentage to acid-labile phosphorus and ordinate, the enzyme concentration in logarithmic scale. $-\text{Log}_2$ O of B and C corresponds to 970 μg. of protein nitrogen in 1.8 ml. of reaction system, and of A to 370 μg. A is the curve for the purified hexokinase preparation, C is a two-step curve for crude hexokinase preparation containing a considerable amount of adenylate kinase, and B for that containing a small amount of the kinase.

concentrated enzyme preparation, it is anticipated that the acid-labile phosphorus of ADP may be incorporated into glucose due to a coupled reaction of these enzymes. For its confirmation, incorporation of ADP as a substrate into glucose was investigated (Table I).

Several samples of ATP in the purification process were estimated on the values of chemical components and Inco.-P and the results are shown in Table II. Pentose was determined by the method of Mejbaum (*24*). Adenine was determined by measuring the ultraviolet absorption at 258mμ (absorption maxima in pH 7.0).

As can be seen in Table II, it was confirmed that the values of Inco.-P

are proportional to the amount of ATP contained in the sample used. An application of this method to the determination of ATP in physiological materials was examined as follows. Rabbit was killed by the injection of

TABLE I

Dependence of Inco.-P on the Concentration of Crude Hexokinase

	Conditions in the reaction systems			Inco.-P
	Substrate	Acid-labile phosphorus of substrate	PH	
		(*μ moles*)		(*μ moles*)
Concentrated enzyme	ADP	0.47	5.65	0.22
	,,	,,	6.60	0.34
	,,	,,	7.28	0.38
	,,	,,	7.94	0.46
	ATP	2.10	6.95	1.48
	,,	1.85	,,	1.35
Diluted enzyme	ADP	0.55	6.95	0.00
	,,	0.39	7.28	0.00
	ATP	1.87	6.95	0.90
	,,	1.85	,,	0.91
	,,	1.77	,,	0.79

The enzyme preparation used is corresponded to curve C in Fig. 1, the upper 970 *μ*g. of protein nitrogen in 1.8 ml. of reaction system, the lower 180 *μ*g. of that. Other conditions are described in the text.

TABLE II

Analyses of ATP in the Purification Process

	Ortho-phosphate	Acid-labile phosphorus	Total phosphorus	Pentose	Adenine	Inco.-P.	Purity (%)
Crude ATP	0.32	1.04	2.35	0.78	0.68	0.45	45
ATP crystallized	0.22	1.61	2.86	1.00	0.87	0.57	57
ATP recrystallized	0.00	1.89	3.09	1.05	0.97	0.90	90
ATP recrystallized two times	0.00	1.93	2.99	1.01	1.01	0.96	96
Theory of ATP	0.00	2.00	3.00	1.00	1.00		

The values are expressed in micromoles per a micromole weight of samples. In the assay system 0.1ml. of *M*/100 ATP was used. Analytical method are described in the text. Adenine was calculated from the optical density at 258 m*μ*. by 1.42×10^4 of its molar extinction coefficient (*26*).

magnesium sulfate. The organs were rapidly removed, weighed, and were powdered by crushing and grinding with dry ice. Perchloric acid was added until the concentration reached $0.25\sim0.30\,N$ ($0.5\,N$ on the blood). The

suspension was placed in an ice bath for 2 hours and then centrifuged. The extraction was repeated three times. Then each of the supernatant fluids was neutralized with KOH and cleared by discarding $KClO_4$ crystals. The Inco.-P, acid-labile phosphorus, and pentose were determined in each of these protein-free filtrates.*

As shown in Table III, a large amount of Inco.-P was found in the heart, stomach, skeletal muscle, and blood, but little or none in the brain, liver, and kidneys. It may be assumed that some amounts of ATP were lost during the preparation procedure.

The reliability of the determination of ATP in physiological samples was checked by measuring the recovery of ATP added to an extract of muscles (Table IV).

From the results in Table II, which indicate that almost one-half of

TABLE III

ATP Analyses on Rabbit Organs and Blood

Materials	Inco.-P (A)	Acid-labile phosphorus (B)	Ortho-phosphate	Pentose (C)	$\frac{(B)}{(C)}$	$\frac{2\times(A)}{(B)}$
	(*μ moles*)	(*μ moles*)	(*μ moles*)	(*μ moles*)		
Heart muscle	0.89	3.26	11.49	7.29	0.45	0.54
Liver	0.02	2.51	11.35	11.54	0.22	0.02
Kidney	0.04	1.70	8.76	6.32	0.28	0.04
Stomach muscle	1.15	3.13	9.71	5.99	0.52	0.74
Skeletal muscle	5.69	11.93	31.69	9.43	1.27	0.96
Brain	0.00	1.31	14.33	4.25	0.31	0.00
Blood	0.37	0.71	0.80	0.77	0.92	1.04

The value are expressed per g. or ml. of fresh material, and calculated from the sum of the infinite geometrical progression of the three estimated values, as described in the text.

acid-labile phosphorus of pure sample of ATP is incorporated into glucose, and the data of recovery test with the physiological material (Table IV), it is reasonable to conclude that the Inco.-P represents the amount of ATP. In the results shown in Table III, it will be noticed that no correlation exists between the amount of Inco.-P and acid-labile phosphorus.

Furthermore, examinations were made with the extracts by 0.25~0.30 *N* $HClO_4$ of young mycelial mats and mature mats of *Aspergillus oryzae*.

ATP was found, though in a small amount, in the extract of the mature mats but not in that of young one, which is indicated in Table V. It may be considered that inorganic polyphosphate in extracts inhibited hexokinase reaction (*25*). It will be reported elsewhere on the phosphorus metabolism

* When a large amount of orthophosphate is present, it is desirable to remove it previously, *e.g.* by using anion exchange resin.

of *Aspergillus oryzae*, and the correlation between the growth and acid-labile phosphorus compounds contain some interesting facts.

Because the method mentioned above for the determination of ATP in biological materials is more useful than a chemical one, it was compared with another enzymatic method for the purpose of evaluation. Berger *et al.* (*16*) determined hexokinase activity by a manometric measurement of the CO_2-evolution from a bicarbonate buffer. The method is based on the fact that one acid equivalent is formed per mole of phosphorus transferred from ATP

TABLE IV

Recovery Test of ATP in Muscle Extract

Sample in assay system with hexokinase	Ortho-phosphate	Acid-labile phosphorus (A)	Inco.-P (B)	$\frac{2\times(B)}{(A)}\times 100$
	(μg.)	(μg.)	(μg.)	
(a) Muscle extracts (1 *ml.*)	66.2	44.1	21.0	95.4
(b) ATP solution (0.1 *ml.*)	1.8	27.5	13.7	99.7
(c) Muscle extracts 0.5 *ml.* plus ATP solution 0.1 *ml.*	34.9	49.9	24.0	96.3
Recovery* (%)	100.0	101.3	98.5	

Analytical conditions for calculation of Inco.-P are described in the text.

* Calculated; $\frac{(c)-(a)\times 1/2}{(b)}\times 100$

TABLE V

ATP Content in Extracts from Mycelial Mats of Aspergillus oryzae

	Acid-labile phosphorus (A)	Inco.-P (B)	pentose (C)	$\frac{(A)}{(C)}$	$\frac{2\times(B)}{(A)}$
	(μ *moles*)	(μ *moles*)	(μ *moles*)		
Young mats	1.07	0.00	0.50	2.14	0.00
Mature mats	1.05	0.23	0.36	2.92	0.44

A,B, and C are expressed per mg. of nitrogen. Nitrogen in g. of fresh weight is 4.9 mg. in young mats grown for 20 hours in Pfeffer' medium at 30° and 7.5mg. in mature mats grown for 70 hours under the same conditions.

to glucose. A method according to this principle was attempted by the authors for the assay of ATP. Consequently, a linear correlation was obtained within 1~8 micromoles of ATP (±0.3 micromoles in accurancy). From the comparison of the values obtained by this method with the Inco.-P values (Table VI), it was found that the manometric method is less suitable than the latter unless the samples can be used in a large amount.

On the other hand, Slater (*5*) reported a spectrophotometric determination of hexose monophosphate, hexose diphosphate, ATP, and ADP

using glycolytic enzymes prepared from skeletal muscle. These were determined by disappearance of DPNH by detection of optical density at 340mμ in the reaction of α-glycerophosphate dehydrogenase. The method was also confirmed by rhe present authors. Then the values were calculated from the standard curve with purified G6P in comparison with the Inco.-P values which are shown in Table VII. The method is too complicated to measure the amount of ATP, though it is capable of determining 0.1 micromoles of ATP in the sample used.

The enzymatic methods for determination of ATP has been published by many other workers. Some methods using ATP-ase are simple and handy to use, though the enzyme prepared must be of high purity and of strict specificity. In the case of the method using phosphorylation system, ATP is detected in correlation with the metabolic function and such strictness will not be required, but the method requires complicated factors in determining phosphorylated products. According to the manometric method it is possible to determine directly the acid hydrogen ions of G6P formed proceeding the hexokinase

TABLE VI

Comparison of the Values Obtained by Manometric Method with Inco.-P

Experimental No.	Inco.-P	Manometric method
	(*μmoles*)	(*μmoles*)
1	1.21	1.57
2	2.90	2.96
3	4.45	4.35
4	6.12	6.46

TABLE VII

Comparison of the Values in Slater's Method Using G6P As a Standard with Inco.-P

Experimental No.	Inco.-P	Recovery as G6P
	(*μmoles*)	(*μmoles*)
1	0.52	0.56
2	0.87	0.95

reaction, but this method cannot be applied, owing to lower detection value, when the reaction mixture is accompanied by buffering action or acid producing process.

Compared with these, the method reported in this paper, which determines ATP by measuring decrease of acid-labile phosphorus, is convenient in obtaining directly the value of ATP and does not require complicated factors.

SUMMARY

1. For the determination of ATP, a simple reaction of hexokinase prepared from yeast was employed by measuring diminution of acid-labile phosphorus. Compared with two other methods, manometric and spectrophotometric, using hexokinase, the present method is easy to use.

2. Illustrative examples are given of analysis of ATP in pure solution and in extracts from physiological materials. The results obtained from these experiments, especially in the latter case, make it quite clear that the acid-labile phosphorus is not the representative of ATP, but phosphorus

incorporated into hexose is compatible with the content of ATP.

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The authors wish to express many thanks of the Oriental Yeast Ltd. for their kind supply of the pressed yeast.

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A NEW METABOLITE OF STREPTOMYCES ETHOXY-ETHENE-1, 2-DICARBOAMIDE

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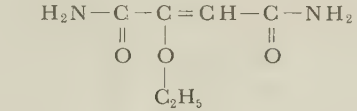
(Received for publication, August 2, 1957)

Further studies on the metabolite produced by *Streptomyces* No. 902 revealed that this organism accumulated a new metabolite, ethoxy-ethene-1, 2-dicarboamide (I) considered as an ethylated derivative of enol-oxalacetdiamide under proper cultural condition*. The structural studies on the metabolite were herein reported.



(I)

From the cultured broth filtrate of approximate 24 hours age, the isolation procedure described in the following paper led to the particular metabolite having the molecular formula fitted for $C_6H_{10}N_2O_3$; d.p. 220–222° on alminium block.

The analytical data of the sample dried on P_2O_5 under 10^{-1} mm. Hg at 14° over night show: C, 45.53 per cent; H, 6.19 per cent; N, 17.57 per cent, and the molecular weight by Rast method with phenol falls in with 156. Calculated for $C_6H_{10}N_2O_3$: C, 45.63 per cent; H, 6.38 per cent; N, 17.72 per cent and molecular weight, 158. The metabolite is optically inactive in water. The ultraviolet absorption spectrum in water shows the maximum at 226 mμ ($E^{1\%}_{1cm}=1288$) as in Fig. 1, and supports the suggestion that the compound is an aliphatic one having an unsaturated bond conjugated with carbonyl group under some bathochromic effect. The infrared absorption spectrum in nujol shows maxima, as in Fig. 2, at 2.92, 3.11, 6.04, 6.20 and 7.12 microns assigning the amide carbonyl group and at 6.04 with 6.20 microns assigning a double bond conjugated with carbonyl group. And further more, the absorption at 9.19 microns seems to assign the presence of etheric C-O bond, and the absorption at 6.20 and approximate 12.12 microns seem to assign the presence of tri substituted olefine. The presence of nitrile or diazo group is excluded by the infrared absorption spectrum. Ninhydrin, biuret and anthrone tests are negative. The detection tests for nitro or oxime group are negative. The degradation product by 1 *N* NaOH in a boiling water bath gives a blood-

* The cultural condition is the same as in the case of the production of trans-cinnamic acid amide by this organism already reported (*1*) and will be described in the following paper.

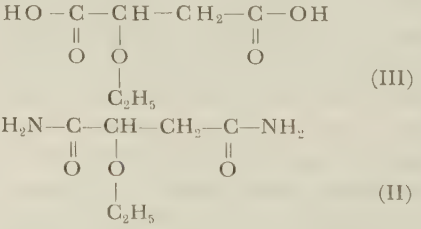
red color reaction with 1 per cent $FeCl_3$ aqueous solution after neutralization with 1 *N* HCl. The color reaction is very similar to oxalacetic acid.

The reduction by 10 per cent palladium charcoal catalyst in methanol gives rise to the product (II) $C_6H_{12}N_2O_3$ showing m.p. 178–182° after the uptake of 1 mole of hydrogen per mole of substrate. The ultraviolet absorption spectrum in water shows a drastic diminution in the strong absorption at 226 mμ of the substrate and shows no characteristic absorption. The infrared absorption spectrum in nujol shows maxima as in Fig. 3 at 2.97, 3.11, 5.96, 6.12 and 7.07 microns assigning amide carbonyl group and at 8.10 and 8.90 microns assigning etheric C-O bond.

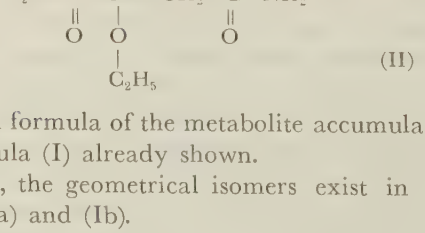
The hydrolysis of the reduction product (II) by 20 per cent sulfuric acid in a boiling water bath gives rise to the product (III) $C_6H_{10}O_5$ showing m.p. 88–94° (unsharp). R_f by *n*-butanol—formic acid—water system (4 : 0.7 : 1 *w/w*) is 0.73. The infrared absorption spectrum in nujol shows that this compound is a typical carboxylic acid probably having etheric C-O bond. The sublimation of the hydrolysis product under high vacuum gives rise to the product (IIIa) showing m.p. 86–88°. Its molecular weight determined by titration falls in with 162 as a dibasic acid. The R_f value and the infrared absorption spectrum of the product (IIIa) quite agree with those of the hydrolysis product (III). So these two compounds (III and IIIa) are identical.

The degradation of the hydrolysis product by conc. hydrochloric acid in a closed tube at 120° for 1 hour gives rise to the product (IV) having R_f of 0.49 by *n*-butanol—formic acid—water system (4 : 0.7 : 1 *w/w*). The degradation product quite agrees with DL-malic acid in the paperchromatographic analysis under the same conditions described above, and the Ca-salt of the degradation product and of DL-malic acid are identical in the infrared absorption spectra in nujol as in Fig. 5.

According to the above experimental evidences, the structural formula of the hydrolysis product (III) is given as the following formula (III), and then the formula of the reduction product (II) is given as the following formula (II).



(III)

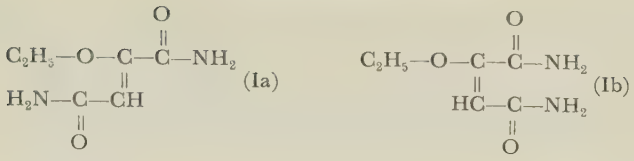


(II)

So the structural formula of the metabolite accumulated by this organism is given as the formula (I) already shown.

Generally saying, the geometrical isomers exist in such a structure as following formula (Ia) and (Ib).

But the geometrical isomerism status in this naturally occuring metabolite was not established in these studies.



(Ia) (Ib)

EXPERIMENTALS AND RESULTS

I. The Ultraviolet and Infrared Absorption Spectra of the Metabolite

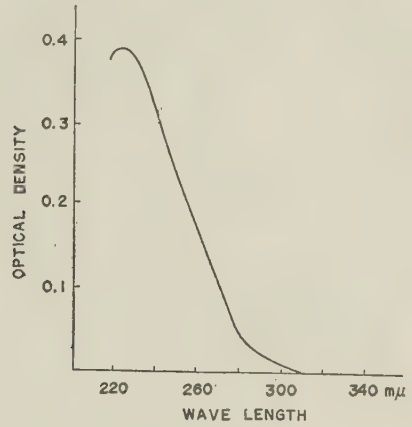


Fig. 1. The ultraviolet absorption spectrum of the metabolite $C_6H_{10}N_2O_3$ in water.

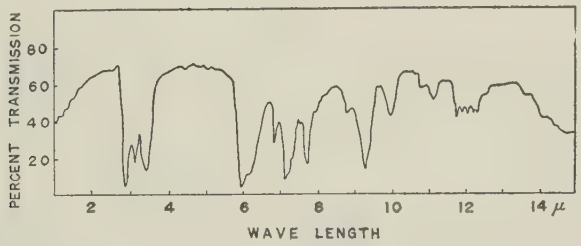


Fig. 2. The infrared absorption spectrum of the metabolite $C_6H_{10}N_2O_3$ in nujol.

II. The Catalytic Hydrogenation of the Metabolite by 10 per cent Palladium Charcoal Catalyst—100 mg. of the metabolite in 40 ml. of methanol is hydrogenated using 70 mg. of 10 per cent palladium charcoal catalyst. The catalyst is removed by filtration. The filtrate is concentrated under diminished pressure until the reduction product is crystallized. The crystal is gathered by filtration and then washed by water and ethanol before drying. Yield; 62 mg. The product after drying on P_2O_5 at 14° under 10^{-1} mm. Hg over night melts at about 178–182° on alminium block. Found: C, 44.53 per cent;

H, 7.25 per cent; N, 18.10 per cent, molecular weight by Rast method with phenol falls in with 160. Calculated for $C_6H_{12}N_2O_3$: C, 44.97 per cent; H, 7.55 per cent; N, 17.50 per cent and molecular weight, 160.

The results obtained by the conventional Warburg's apparatus using mercury in a manometer are as following.

TABLE I

The Catalytic Hydrogenation of the Metabolite by 10 per cent Palladium Charcoal Catalyst

Sample (*mg.*)	H_2 up-take (*ml.*)	Time to cease H_2 up-take (*mins.*)	Theoretical molecular weight	δ
5.124	0.802	350	158	1.1
11.211	1.664	380	158	1.0

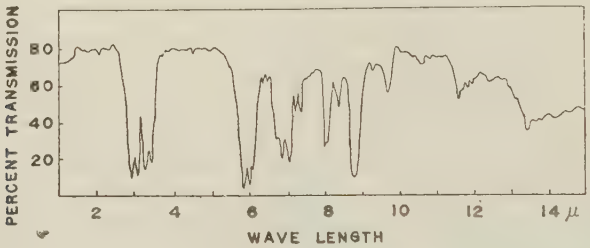


FIG. 3. The infrared absorption spectrum of the reduction product $C_6H_{12}N_2O_3$ in nujol.

III. The Hydrolysis of the Reduction Product by 20 per cent Sulfuric Acid—45 mg. of the reduction product is dissolved in 10 ml. of 20 per cent sulfulic acid aqueous solution and heated in a boiling water bath for 8 hours under a reflex condenser. The hydrolysate solution is repeatedly extracted by the same volume of ethylacetate. After drying the solvent with anhydrous sodium sulfate, ethylacetate is removed until the product is crystallized. Yield: 31 mg. m.p. 88–94° (unsharp). Found: C, 44.16 per cent; H, 6.07 per cent and no presence of nitrogen. The molecular weight estimated by titration falls in with 160 as a dibasic acid. Calculated for $C_6H_{10}O_5$: C, 44.42 per cent; H, 6.22 per cent and molecular weight, 162. The R_f by *n*-butanol—formic acid—water system (4 : 0.7 : 1 *w/w*) is 0.73. The detecting reagent in the paperchromatographic analysis is 0.1 per cent B.P.B. ethanolic solution. The purification of this organic acid is effectively performed by sublimation under 3×10^{-1} mm. Hg at about 100°. The sublimation product melts at 86–88° and is quite identical with the above hydrolysis product in the infrared spectroscopy and in the paperchromatographic analysis. The molecular weight determined by titration matches to 162 as a dibasic acid.

IV. The Conversion of the Hydrolysis Product to DL-*Malic Acid*—20 mg. of

the hydrolysis product is dissolved in 3 ml. of conc. hydrochloric acid and is sealed in a glass tube. The sealed tube is heated at 120° for 1 hour in an

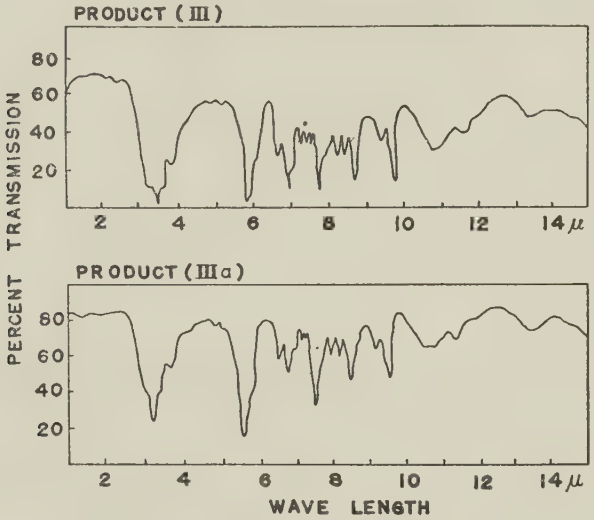


Fig. 4. The infrared absorption spectra of the hydrolysis product (III) and the sublimation product (IIIa).

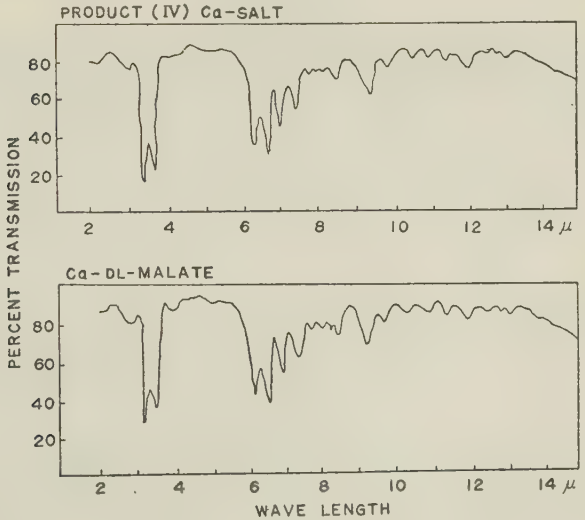


Fig. 5. The infrared absorption spectrum of the Ca-salt of the degradation product (IV) comparing with Ca-DL-malate.

autoclave. The resulted solution is concentrated under vacuum to yield dryness. The dryness is repeatedly concentrated under vacuum adding

water to remove hydrogen chloride from the dryness. Calcium oxide is gradually added to the aqueous solution of the dryness until the neutral reaction is indicated. The precipitation of Ca-salt of the degradation product is gathered by filtration and washed by water. And then it is dried under 10^{-1} mm. Hg at 80° 8 hours. Yield: 17.6 mg. Found: Ca, 22.82 per cent. Calculated for $C_4H_4O_5Ca$: Ca, 23.34 per cent. R_f by *n*-butanal—formic acid—water system (4: 0.7: 1 *w/w*) is 0.49 when applicated as 10^{-1} *N* hydrochloric acid solution and any other acidic substance is not detected excepting hydrochloric acid at R_f 0.12. The R_f value and the infrared absorption spectrum of the Ca-salt quite agree with those of Calcium DL-malate. From the melting point of the above hydrolysis product (III) (*2*), it is presumed that the malic acid obtained is racemic form.

SUMMARY

The metabolite having the molecular formula fitted for $C_6H_{10}N_2O_3$ obtained from the cultured broth filtrate of *Streptomyces* No. 902 was decided as a new metabolite of *Streptomyces*, ethoxyethene-1, 2-dicarboamide.

Author gratefully thanks to Prof. Dr. Y. Nakamura for his many kind advices, also to Assist. Prof. Dr. S. Tamura for the infrared spectroscopy and Mrs. Y. Baba for the micro elementary analyses. Author was also indebted to Mr. M. Arishima for his advices in microbiological field, and to Mr. M. Ogasawara for pilot plant fermentation and to Mrs. K. Harada for the skilful assistance.

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STUDIES ON THE DENATURATION OF LYSOZYME

II. UREA DENATURATION

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In the previous papers, the author reported the surface denaturation (*1–4*) and the heat denaturation of lysozyme (*5*). It was found that lysozyme has many interesting features from the viewpoint of denaturation. In this paper will be described the results on the urea denaturation obtained by the viscosity measurements. The result obtained by the present experiments show the remarkable correspondence with those by the surface chemical method which were reported in the previous papers. This paper will be also concerned with the mechanism of urea denaturation of lysozyme ann with the consideration of native structure of lysozyme approached by the studies of the various denaturation which have been so far carried out. Hitherto, the report on the denaturation and inactivation of lysozyme by urea was only given by Lénois (*6*).

EXPERIMENTALS

Lysozyme was prepared from hens' egg white by direct crystallization method and recrystallized four times and finally lyophilized. Urea was recrystallized three times from 70 per cent ethyl alcohol.

The viscosity of lysozyme solutions was measured by means of Ostwald viscometer. The flow time of water was 189.6 seconds at 25.0°. The densities of the solutions were determined by Ostwald pycnometer. Reduced viscosity(η_{red})was calculated by equation (1).

$$\eta_{red}=\frac{\eta_{sp}}{c}=\frac{\eta_r-1}{c} \qquad (1),$$

where η_{sp} is the specific viscosity, η_r the relative viscosity and c the concentration of lysozyme expressed by grams of the protein per 100 ml. The concentration of lysozyme was determined by micro-Kjeldahl method.

RESULTS

The change with time of the reduced viscosity of lysozyme in the solutions of various concentrations of urea at various temperatures are shown in Fig. 1. The concentration of lysozyme was 0.41 g./100 ml. Acetate buffer was used and the pH's varied between 5.4 and 5.8 depending on the concentration of urea.

It is to be noted in Fig. 1 that the reduced viscosity was increased instantaneously with the rise of temperature and did not change with time at

each temperature. The small effect of time was observed between 35° and 45° in 10 M urea and at 50° in 9.2 M urea. The change with time of the reduced viscosity in 10 M urea at 45° was also shown in Fig. 1. In this case the rapid increase of viscosity was observed for the first 30 minutes after which

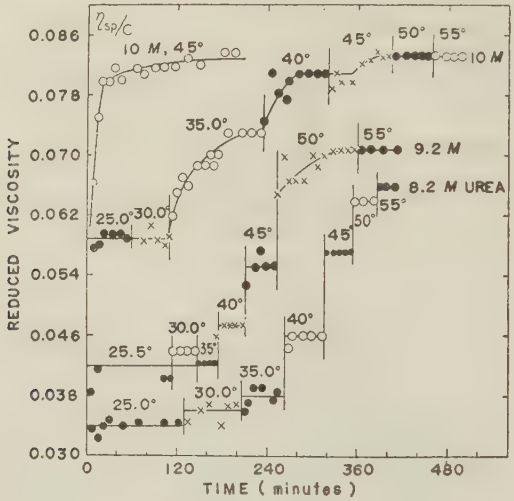

Fig. 1. The change with time of reduced viscosity of lysozyme Concentration of lysozyme: 0.41 g./100 ml. pH 5.4—5.8.

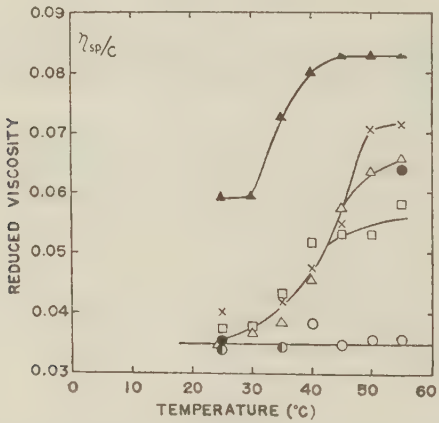

Fig. 2. Relationship between maximum reduced viscosity and temperature. ◐ : 0 M, ○ : 4.2 M, □ : 6.3 M, ● : 7.4 M, △ : 8.2 M, × : 9.2 M, ▲ : 10 M urea.

the viscosity attained to the final value at the same level at 45° which was obtained by the succesive rise of temperature from 25°.

Fig. 2 shows the maximum values of the reduced viscosities at each temperature.

It was found from Fig. 2 that the reduced viscosity in 4.2 *M* urea did not change between 25° and 55° and was the same as that of native lysozyme. Above 6.3 *M* urea, the reduced viscosity increased from about 30° and tends to approach the constant value from about 45°. Although the viscosity-temperature curves were almost the same at lower temperatures, the viscosity became greater with the increase in concentration of urea at higher temperatures. The reduced viscosity in 10 *M* urea was greater than that in 9.2 *M* urea even at 25°. In this case the viscosity was also increased from 30° and became constant at and above 45°.

After the lysozyme solution once heated up to 55° was stood at room temperature overnight, the viscosity was measured again at 25°. The results are showd in Table I. In 10 *M* urea, the reduced viscosity thus obtained was the same as that 55°. The viscosity returned to the original value

TABLE I

Reversibility of Lysozyme Denaturation in Urea Solutions

Urea concentration (*M*)	Temperature (°C)	η_{sp}/c
10	25.0	0.059
	55	0.083
	25.0 (recovered)	0.083
9.2	25.5	0.040
	55	0.072
	25.5 (recovered)	0.051
8.2	25.0	0.035
	55	0.066
	25.0 (recovered)	0.042

at 25° to a smaller extent for 9.2 *M* urea. In 8.2 *M* urea the reversibility of the viscosity was nearly complete.

The reversibility of urea denaturation of proteins is usually tested by the removal of urea by dialysis. In the case of lysozyme, however, the reversibility was observed merely by the lowering of temperature. It might be appropriate to term these phenomena as the "heat denaturation of lysozyme in urea solution" in place of "urea denaturation".

The effects of pH's and of the splitting agent for disulfide bonds, sodium sulfite, on the viscosity-time curve are shown in Fig. 3. The concentration of lysozyme was 0.41 g./100 ml. and the urea concentration was 8.2 *M*. Acetate buffer (*N*/10) and borate buffer (*N*/20) were used for pH 5.6 and 9.6, respectively. It is evident from this figure that the values of reduced viscosities at pH 9.6 were higher than that at pH 5.6. At pH 9.6, the reduced viscosity increased instantaneously at 30° and at 35° and did not change with time. At 40°, however, the reduced viscosity changed with time. In 8.2 *M* urea at

pH 9.6 containing 0.03 *M* sodium sulfite, the reduced viscosity was increased rapidly with time and the final value was much higher than that in the

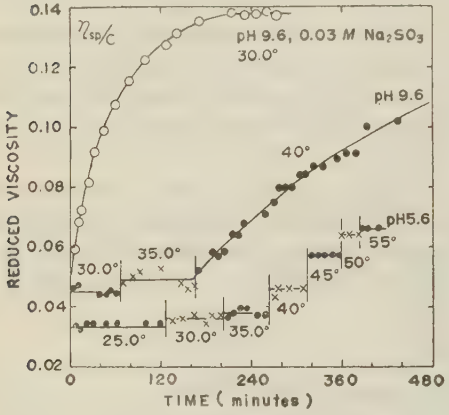

Fig. 3. Effect of pH and of sodium sulfite (0.03*M*) on reduced viscosity. Urea concentration : 8.2 *M*.

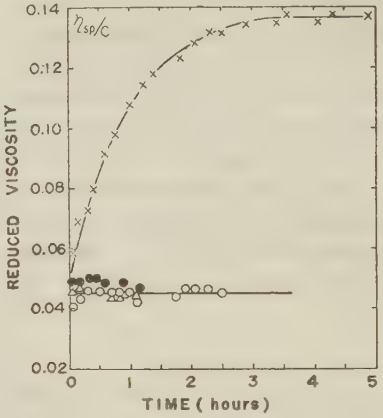

Fig. 4. Effect of sodium sulfite on reduced viscosity at different urea concentrations. 30.0°, pH 9.6. ● : 6.3 *M* urea, ○ ; 6.3 *M*. urea, 0.03 *M* Na_2SO_3, △ : 8.2 *M* urea, × : 8.2 *M* urea, 0.03 *M* Na_2SO_3.

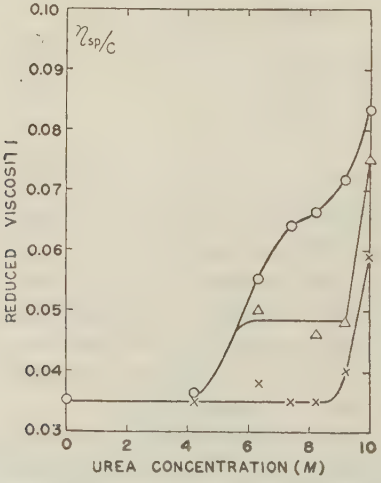

Fig. 5. Relationship between reduced viscosities at each temperature and urea concentrations. × : 25.0°, △ : 40°, ○ : 55°. pH 5.4—5.8.

absence of sodium sulfite at 30°. The curve at pH 5.6 was the same as that shown in Fig. 1.

Fig. 4 illustrates the effect of sodium sulfite (0.03 *M*) at different urea

concentrations. Fig. 4 clearly shows that the effect of sodium sulfite was not evident in 6.3 *M* urea but was much striking in 8.2 *M* urea. In the absence of sodium sulfite, however, the reduced viscosity was almost the same irrespective of the urea concentrations.

The reduced viscosities at each temperature were plotted against urea concentrations in Fig. 5.

DISCUSSION

Comparison with the Results Obtained by Surface Chemical Method

In the previous papers (*1*, *2*) we had some insights into the mechanism of urea denaturation of lysozyme by surface chemical method. The surface chemical method was based on the facts that the expansion of lysozyme monolayers is profoundly affected by whether they are spread from a solution of native lysozyme or from the urea solution in which lysozyme is denatured by urea. The results obtained by surface chemical method are summarized briefly as follows:

(1) The area per residue of the lysozyme monolayer spread from 5 *M* urea solution was the same as that of native lysozyme.

(2) The maximum expansion of the monolayer was obtained when it was spread from 10 *M* urea solution.

(3) In the case of 8 *M* urea the dependence of the expansion on the temperature of substrate was the same as that of native lysozyme except that the expansion was greater if compared at the same temperature.

It is interesting to compare these results with those obtained by the present experiments. The results obtained by the viscosity measurements were as follows: In 4 *M* urea the reduced viscosity did not change between 25° and 55° and was the same as that of native lysozyme. In 8.2*M* urea reduced viscosity was increased with the rise of temperature. In 10 *M* urea the reduced viscosity was much higher than those in 8.2 *M* urea.

The following may be inferred from the comparison with the results obtained by these two different methods.

(1) Lysozyme is not denatured in the solution of urea concentrations below 4 or 5 *M*.

(2) The denaturation in 8 *M* urea may be caused by rather slight change in the tertiary structure of lysozyme molecule. This may be seen from the facts that while the area occupied by the lysozyme monolayer spread from 8 *M* urea was greater than that spread from a native lysozyme, the temperature dependence of the expansion was the same as that of native lysozyme, that the viscosity of the solution once heated up to 55° returned to the original value at 25°, and that no change with time of reduced viscosity was observed. The denaturation of lysozyme in 8 *M* urea is very interesting and such a phenomenon has not been observed for other protein denaturation, the details of which will be discussed again in the later part of this paper.

(3) The secondary structure of lysozyme molecule may be destroyed above 9 *M* urea. This is supported from the facts that the maximum expansion

was obtained for the monolayers spread from 10 M urea, that the reduced viscosity in 10 M urea was much higher than that in 8 M urea, and that the reduced viscosity did not reverse to the original value by cooling.

The Behavior of Lysozyme in 8 M Urea

As mentioned above, it was found that in 8 M urea (pH 5.6) the viscosity dropped reversibly by cooling, that the change of the viscosity with time was not observed at each temperature and that the viscosity tends to approach the constant value above 50°. From these facts it is assumed that at each temperature native lysozyme molecules are in equilibrium with those of another modification which are stable above 50°. The equilibrium constant

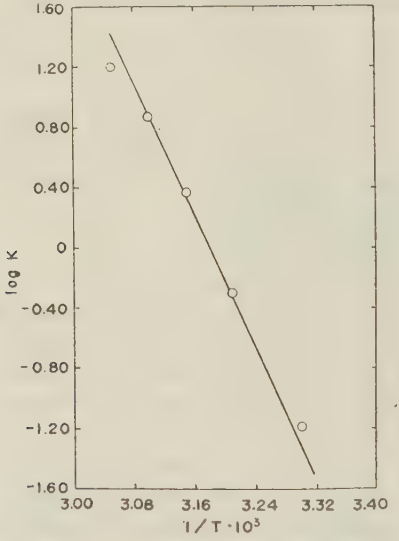

Fig. 6. Relationship between logarithms of equilibrium constants (K) and reciprocals of absolute temperatures.

(K) at each temperature may be calculated from equation (2).

$$K=\frac{\eta-\eta_0}{\eta\infty-\eta} \qquad (2)$$

where η_0 is the reduced viscosity of native lysozyme and $\eta\infty$ the viscosity above 50°.

The relation plotted the logarithms of the equilibrium constants against the reciprocal of absolute temperature is straight line as shown in Fig. 6.

Then, the change in enthalpy can be evaluated from van't Hoff's equation. The change in standard free energy (ΔF°) and entropy (ΔS°) can be also obtained by the usual thermodynamic calculations. These thermodynamic parameters are shown in Table II.

The changes in enthalpy and entropy shown in Table II are much smaller

than those generally accepted for protein denaturation. With the reversible heat denaturation of soybean trypsin inhibitor, for instance, $\Delta F°=87.5$ cal./mole, $\Delta H°=57{,}300$ cal./mole, $\Delta S°=180$ e.u. (at 45°) (*7*). These facts also support the above mentioned considerations that lysozyme molecule suffers only small structural change in 8 *M* urea. According to Léonis (*6*), the change in optical rotation in 8 *M* urea is only 8 per cent, indicating the small change in the folded structure*.

The Role of Disulfide Bonds in the Urea Denaturation of Lysozyme

The reduced viscosity in 10 *M* urea (pH 5.8) heated to 55° was only 0.083. This value is much smaller than that for other urea denatured proteins and is comparable with that for ribonuclease in 8 *M* urea. In the case of ribonuclease the intrinsic viscosity in 8 *M* urea is 0.089 (*8*). This may be caused by the fact that many disulfide bridges are present in these rather small molecules and prevent the uncoiling of the molecule. Previously, it was shown that the monolayer properties are affected profoundly by the presence of the splitting agents such as sodium sulfite or cyanide (*4*).

TABLE II

Thermodynamic Parameters of Lysozyme Denaturation in 8 M Urea (45°)

$\Delta F°$ (*cal./mole*)	$\Delta H°$ (*cal./mole*)	$\Delta S°$ (*e. u.*)
−527	4,946	17.2

As shown in Fig. 4, the reduced viscosity was much increased with time and attained to the value of 0.137. This value is the same order as that of other proteins in urea solutions. This fact shows that the lysozyme molecules cannot be extended merely by the urea denaturation but can be uncoiled by the splitting of the disulfide bonds. As suggested by Frensdorf, Watson and Kauzmann (*9*) in the case of urea denaturation of serum albumim, the five intrapeptide disulfide bridges of lysozyme must be also incorporated into widely separated parts of polypeptide chains and they are not neighbors along the chains.

In the case of 6.3 *M* urea, sodium sulfite did not affect the reduced viscosity. This fact shows that the disulfide bridges might be masked in the interior of the molecules and that the degree of denaturation in 6.3 *M* urea is so small that the disulfide bonds cannot react with sulfite. Fraenkel-

* In the previous paper (*2*), the greater expansion of lysozyme monolayer spread from *8 M* urea than that of native lysozyme was considered to be due to the lowering of activation energy which is necessary in order to obtain the monolayer consisting of completely unfolded molecules. However, as convinced from the above discussion, the following views are more plausible: In *8 M* urea an equilibrium exists between native and reversibly denatured lysozyme molecules. Therefore, the greater expansion of the monolayer spread from 8 *M* urea is due to these reversibly denatured molecules which are more susceptible to surface denaturation.

Conrat, Mohammad, Ducay and Mecham (*10*) have also shown that the disulfide bonds of lysozyme could be reduced only in concentrated urea solutions and that the reducing the disulfide bonds was of all-or-none character. The easiness of the splitting of disulfide bonds gives a measure of degree of denaturation by urea.

The change in reduced viscosity with time was observed when the solution in 8.2*M* urea (pH 9.6) was heated at 40° (Fig. 3). This might be also due to the splitting of disulfide bonds owing to the alkaline pH (*11*).

Survey of the Denaturation of Lysozyme in Relation to Its Structure

As pointed out by Kauzmann (*12*) and by Lumry and Eyring (*13*), the native structure of proteins is consisted of primary, secondary and tertiary structure. Kauzmann (*14*) also described the following five intramolecular bonds which might be important in maintaining the native protein in its organized structure: disulfide bonds, hydrophobic bonds, salt bridges, hydrogen bonds, and London dispersion forces.

The concern, hare, is with what level of these primary, secondary, or tertiary structure is affected by each type of denaturation and with what groups are essential in maintaining the native lysozyme molecule.

Lysozyme is more unstable in alkaline medium in both the urea- and the heat-denaturation. The isoelectric point of lysozyme lies near pH 11. If native lysozyme structure were maintained by salt-like bonds, the stability of lysozyme must be increased at higher pH's. We may conclude, therefore that in the case of lysozyme, salt bridges might be unimportant for its native structure.

In the case of lysozyme, as pointed out by Tanford ann Wagnen (*15*) the dissociation constant of phenolic groups is much higher and the carboxyl groups are also abnormal. With urea denaturation, peptide hydrogen bonds and the side chain hydrogen bonds are disrupted by urea. The denaturation in 8*M* urea may be caused by the disruption of these side chain hydrogen bonds and not of the secondary peptide hydrogen bonds. As described by Lumry and Eyring (*13*) the destruction of teriary structure without loss of the secondary structure might be reversible. The small values of the changes in enthalpy and entrapy also suggest this fact.

One of the most striking structural features of lysozyme molecule is that five intrapeptide disulfide bridges exist within its small molecule. It is easily imagined that these bridges play an important role on the stability of the native structure. Recently, however, it has been shown that the disulfide bridges have an opposite effect against the stability of folded structure. For instance, Kauzmann claimed this by the measurement of optical rotation of surum albumin. Markus and Karush (*16*) have found that the optical rotation of serum albumin was decreased by the splitting of disulfide bonds, indicating that the folding structure was developed better than the original albumin. Yang and Doty (*17*) also have shown that this is the case for insulin and serum albumin but not for ribonuclease. In the case of lysozyme it is not clear whether such a possibility would exist or not. Lysozyme in

10M urea suffers some change in the secondary structure. The change of viscosity occurred either instantaneously or for a very short time. This fact may reflect the incompleteness of the solded structure. Jirgensons (*18*) found that the optical rotation of lysozyme recovered by dialysis after denaturation by guanidine hydrochloride was lower than that of the original protein. Kauzmann (*12*) explained this fact by a better developed secondary structure than the original native protein. It is seen from the experiments on the effect of sodium sulfite that the disnlfide bonds exist in the interior of the molecule and contribute to the stadility of lysozyme molecule.

The role played by hydrophobic bonds or by London dispersion forces is not clear from our experiments.

Surface denaturation is accompanied with the destruction of the secondary structure. The monolayer in which the secondary structure survive the destruction of the tertiary structure could not be obtained. This is the case for all other protein monolayers. The monolayers in which the secondary structure is stable are those of poly γ-methyl-L-glutamate and poly γ-benzyl-L-glutamate (*19*, *20*).

In the case of heat denaturation of lysozyme, only the tertiary structure may be disrupted and the molecule may aggregate before the secondary structure is changed (*5*).

In the case of urea denaturation, the binding of urea the molecules on the lysozyme molecule must be also taken into consideration (*3*).

SUMMARY

The denaturation of lysozyme in solutions of various urea concentrations over a rather wide temperature range was studied by the measurement of viscosity. The results obtained were as follows:

1. The viscosity of lysozyme in 4 M urea did not change between 25° and 55° and was the same as that of the native protein.

2. Above 6 M urea, the viscosities began to change from about 35° and tended to approach a constant value. This final value was higher with the increase in urea concentration. The viscosity in 10 M urea was much greater than that in 8 M urea at 25°.

3. The effect of time on the viscosity was scarcely observed. Very small change with time of viscosity was observed in 9 and 10 M urea.

4. The effect of pH on the urea denaturation of lysozyme showed that lysozyme was unstable in alkaline medium.

5. In 8 M vrea containing sodium sulfite the viscosity increased greatly with time. In 6 M urea, however, the effect of sulfite was not observed.

6. The results obtained by the present experiments showed the remarkable correspondence with those by the surface chemical method which were reported in the previous papers.

7. Thermodynamic considerations of the denaturation of lysozyme in 8M urea led to the evaluations of the following thermodynamic parameters: $\Delta F^\circ = -527$ cal./mole, $\Delta H^\circ = 4946$ cal./mole, and $\Delta S^\circ = 17.2$ e.u. at 45°. These

values suggest the only minor structural change of lysozyme molecules in 8 *M* urea.

8. The structure of native lysozyme molecule was discussed from the results on the surface-, heat, and urea-denaturation of lysozyme which were so far obtained.

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EFFECTS OF 5′-ADENOSINE MONOSULPHATE AS A SUBSTRATE ANALOGUE ON 5′-ADENYLIC ACID DEAMINASE AND 5′-NUCLEOTIDASE

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It has been shown by Yamashina and Egami (*1*) that 5′-adenosine monosulphate (AMS) inhibits diphosphopyridine nucleotide-linked alcohol dehydrogenase of yeast. In addition AMS was found to compete with flavin adenine dinucleotide and thus inhibit D-amino acid oxidase (Egami and Yagi (*2*)). From these informations it was suggested by these authors that AMS might compete with adenylic acid moiety of these coenzymes. Considering the profound resemblance in structure between AMS and adenosine monophosphate (AMP), it may be quite reasonable to expect that AMS would possibly inhibit enzymes which act on AMP.

The present paper deals with effects of AMS on 5′-adenylic acid deaminase from muscle and on 5′-nucleotidase from potato. The data presented in this paper demonstrate that 5′-adenylic acid deaminase, which is strictly specific for 5′-AMP, was not inhibited by AMS, whereas potato 5′-nucleotidase, a rather poorly specific enzyme, was inhibited by the substrate analogue. Some properties of these enzymes are also described.

MATERIALS AND METHODS

5′-Adenosine Monosulphate—5′ AMS was prepared according to the method originally described by Egami and Takahashi (*3*). The crystalline barium salt was dissolved and converted to the sodium salt by adding an excess amount of sodium sulphate and the resulted barium sulphate was centrifuged off. The solution was stored at 0°.

5′-Adenosine Monophosphate—Disodium salt of AMP used was a chromatgraphically pure preparation from the Sigma Chemical Company.

5′-Adenylic Acid Deaminase—The enzyme was prepared from rabbit muscle by the method of Kalckar (*4*). Lyophilized preparation of the enzyme was turbid when dissolved in 0.01 *M* citrate buffer at pH 6.5, but centrifugation to remove the turbid material resulted in considerable loss of activity from the supernatant. Thus a turbid suspension was used without preliminary centrifugation as the enzyme solution.

Assay of the Deaminase—The deaminase activity was assayed by Kalcker's spectrophotometric method (*5*) based on the 60 per cent decrease in absorption at 265 mμ which occurs on conversion of AMP to inosinic acid. Beckman spectrophotometer (Model DU) and 10 mm. quartz cells were used for optical measurements. The reaction rate was represented

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as ΔE_{265} in a definite reaction period at room temperature (5–10°). Determination of liberated ammonia was carried out with Conway's microdiffusion units by the method of Russell (*6*).

5'-Nucleotidase—5'-Nucleotidase was prepared from potato by the method of Kornberg (*7*). The preparation could be stored in frozen state at −18° for several weeks without any remarkable loss of activity. The precipitates resulted during the storage were removed by centrifugation and a clear supernatant was used as the enzyme solution.

Assay of the Nucleotidase—The nucleotidase activity was assayed by the determination of liberated inorganic phosphate under the following condition; enzyme, substrate, inhibitor and 0.5 ml. of Tris-HCl buffer of pH 9.0 unless otherwise specified in the experimental notes. The reaction temperature was taken at 37° throughout the experiments. The reaction was stopped by the addition of 1.0 ml. of 15 volume per cent. H_2SO_4, and liberated inorganic phosphate was determined according to Allen's method (*8*) omitting the addition of perchloric acid. Optical measurement for inorganic phosphate determination was carried out with Hitachi electrophotometer (Model EPO-A) with R filter (absorption maximum at 670 mμ.).

RESULTS

Effects of AMS on Adenylic Acid Deaminase—A typical reaction sequence of adenylic acid deaminase on AMP is illustrated in Fig. 1. It was also ascertained that the enzyme did not act on AMS at any appreciable rate. Determination of liberated ammonia by Conway's microdiffusion method also proved insusceptibility of AMS as a substrate (Table I).

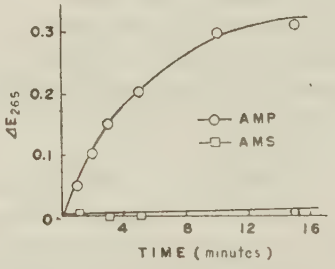

Fig. 1. Action of 5'-adenylic acid deaminase on AMP and AMS. Substrate concentration: AMP, 4.0×10^{-5} *M*; AMS, 3.0×10^{-5} *M*; Citrate. pH 6.5, 0.01 *M*. Enzyme, 0.1 ml. Total volume, 3.1 ml. Temperature, 6°.

In order to check the effect, if any, of the trace amount of barium sulphate which might remain in the AMS solution, influence of added barium chloride was tested, but no inhibition was found even at the concentration of 0.67×10^{-4} *M*. It has been shown by Nikiforuk and Colowick (*9*) that sulphate ion had no effect on the enzyme activity.

No inhibition by AMS was observed as shown in Fig. 2. Because of the absorption of AMS at 265 mμ which is comparable to those of AMP at the same concentration, the amount of added AMS was controlled so as to maintain the total optical density of the reaction mixture at approximately equal level. These data indicate that AMS, even at the concentration of five times

as much as the substrate, does not reveal any inhibition on adenylic acid deaminase.

TABLE I

Assay of Deaminase by Microdiffusion Method

5′-AMP	5′-AMS	Ammonia liberated
μ moles	*μ moles*	*μ moles*
1.4	0	1.5
0	4.4	0.1

50 mg. of lyophilized enzyme preparation dissolved in 5 ml. of 2 per cent NaCl solution was dialysed against 2 per cent NaCl solution for 160 miuntes. 0.3 ml. of this enzyme solution was taken in each Conway's diffusion unit. Reaction time, 24 hours. Temperature, 37°. Total volume, 1.8 ml. Buffer, 0.1 *M* citrate, pH 6.5.

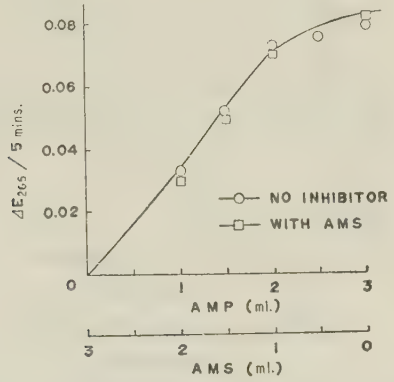

FIG. 2. Effect of AMS on 5′-adenylic acid deaminase. Substrate: AMP, 3.6×10^{-5} *M*. Inhibitor: AMS, 3.0×10^{-3} *M*. Buffer: 0.01 *M*. Citrate, pH 6.5. Enzyme: 0.1 ml. of 10 mg./ml. solution. Temperature, 25°.

Effects of AMS on 5′-Nucleotidase—Barium salt of AMS was found by K. Tanaka in our laboratory to be inhibitory to potato 5′-nucleotidase (unpublished data). In order to avoid influence of barium ion, which strongly inhibits the enzyme, sodium salt of AMS was used in the present experiment. The reaction rate is proportional to the enzyme concentration as shown in Fig. 3. A definite inhibition (33 per cent) was observed in the presence of 1.6×10^{-3} *M* of AMS. Sulphate ion had no effect (Fig. 4). Influence of substrate concentration on the AMS inhibition was also investigated. The effect of increasing substrate concentration on the inhibition by 4.4×10^{-3} *M* AMS is listed in Table II. Increase in the substrate concentration tends to decrease per cent inhibition, but the mode of inhibition by AMS could not be clearly determined.

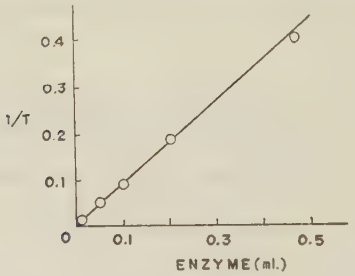

Fig. 3. Effect of the concentration of 5′-nucleotidase on the reaction rate. Reaction mixture: 2.0 ml. of 8×10^{-3} *M* AMP, 2.0 ml. of Tris-HCl buffer 0.056 *M*, pH 9.0, 0.5 ml. of 0.01 *M* $MgCl_2$. Total volume, 5.0 ml. Temperature, 37°. 1/T represents the reciprocal of the time in minute necessary for liberating 20 μg. of inorganic P.

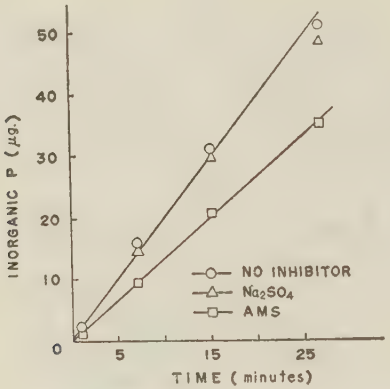

Fig. 4. Effect of AMS on 5′-nucleotidase. Final concentration: AMP, 1.6×10^{-3} *M*; AMS, 4.2×10^{-4} *M*; Na_2SO_4 1.4×10^{-2} *M*; enzyme, 0.1 ml. Temperature, 37°.

Table II

Influence of Substrate Concentration on AMS Inhibition

Final Concentration of AMP	Inorganic P liberated		% Inhibition
	Without AMS	With AMS $4.4\cdot10^{-3}$ *M*	
$3.1\cdot10^{-3}$ *M*	28.8 μg.	13.7 μg.	52%
2.3 //	26.3	11.0	58
1.54 //	23.6	8.2	65
0.77 //	17.3	5.9	66

Enzyme, 0.2 ml. Buffer, Tris-HCl, 0.056 *M*, pH 9.0. Temperature, 37°. Reaction time, 12 minutes.

Influence of pH on AMS Inhibition—As postulated by Dixon (*11*), a remarkable change in inhibition at varying pH would provide some information on active centre of the enzyme as well as the inhibition site. The effects of varying pH on AMS inhibition were studied, but no significant change in per cent inhibition was found as shown in Fig. 5.

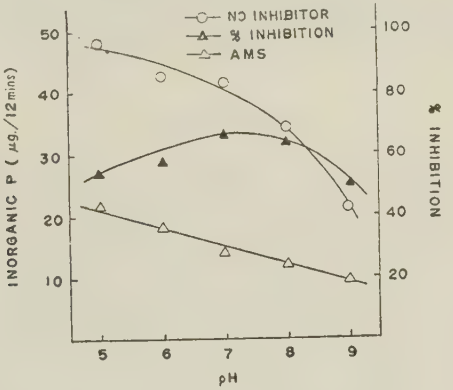

Fig. 5. Effect of pH on activity and AMS inhibition. AMP, $1.6\times 10^{-3}\,M$; AMS, $2.2\times 10^{-3}\,M$; buffer, Tris-Acetate 0.2 *M* enzyme, 0.1 ml. Temperature; 37°.

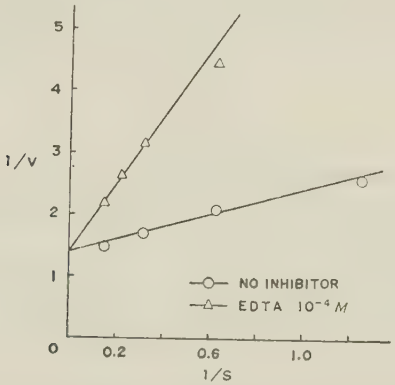

Fig. 6. Effect of substrate concentration on activity and inhibition by EDTA. Substrate concentration (S) expressed as $10^{-3}\,M$, reaction velocity (*V*) as μM per 20 minutes. Enzyme, 0.05 ml. Buffer, 0.056 *M* Tris-HCl pH 9.0. Temperature, 37°.

Inhibition by Adenosine—0.67×10^{-2} and $1.3\times 10^{-3}\,M$ of adenosine inhibited potato 5′-nucleotidase to the extent of 51 per cent and 27 per cent respectively in the presence of $3.3\times 10^{-4}\,M$ of the substrate. Klein (*12*) has also observed such inhibition by adenosine on his purified preparation of the enzyme. The information on the identity of the inhibition site is not yet available.

Effects of EDTA—Addition of $MgCl_2$ (the final concentration 0.1 *M* to 0.0001 *M*) to the enzyme preparation preliminary dialysed against distilled water for 150 minutes failed to demonstrate any requirement of the metal ion for the enzyme activity, but ethylene diamine tetraacetic acid (EDTA) was found to inhibit the enzyme. The mode of inhibition by EDTA investigated according to Lineweaver-Burk's plot (*10*) was found to be competitive one as illustrated in Fig. 6. This result suggests the possibility that a metal firmly bound to the enzyme protein might participate in the enzyme activity.

DISCUSSION

5′-Adenylic acid deaminase is known to be a strictly specific enzyme. The known substrate of the enzyme is only 5′-AMP. All analogues tested such as 2′- or 3′-AMP, ADP, ATP and adenosine have been shown to be insusceptible as the substrate of the enzyme (*4*). In spite of the structural resemblance between AMS and AMP, AMS was neither available as a substrate, nor exhibit any inhibition. This result might be considered as an additional evidence for the remarkably strict specificity of the enzyme.

On the contrary, potato 5′-nucleotidase seems to be poorly specific enzyme. Substrates which have been shown to be hydrolysed by the enzyme preparation include 3′-AMP and 2′, 5′-diphosphate of some ribosides (*7*). It might be quite reasonable to suppose that AMS would probably compete with AMP at the same active site on the enzyme protein. It is of interest to determine the chemical nature of the active centre and of the inhibition site as well as the mode of inhibition by AMS, but further experiments seem to be necessary.

SUMMARY

1. 5′-Adenosine monosulphate (AMS) was found to be ineffective as a substrate of 5′-adenylic acid deaminase from rabbit muscle. No inhibition by AMS was observed on the deaminase.

2. Potato 5′-nucleotidase was shown to be inhibited by AMS. Effects of varying pH's on the inhibition were also investigated.

3. Adenosine and ethylene diamine tetraacetic acid are inhibitory to the 5′-nucleotidase.

The authors are indebted to Mr. A. Asano for his generous collaboration in carrying out the determination of ammonia. The expense of this study was defrayed in part by a grant from Seikagaku-Kenkyujo Ltd., to which our thanks are due.

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BIOCHEMICAL STUDIES ON STREPTOLYSIN S.

III. EFFECTS OF ENZYME DIGESTED RIBONUCLEIC ACID ON THE HEMOLYSIN FORMATION*

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The fact, described in the previous report in this series, that the hemolysin-forming potency of yeast ribonucleic acid (RNA) hydrolyzed with alkali is far inferior to that of yeast RNA hydrolyzed with pancreatic ribonuclease (RNase) in the same degree shows that the high potency of the latter was not only due to the depolymerization but largely due to the specific structure induced by the enzymic hydrolysis (*1–2*).

Since it is to be anticipated that the structure of the active substance that comes into question is not so simple, it seems advisable to investigate first the effects of other enzymic digestion on the hemolysin-forming activity of RNA, leaving the separation of active substance for a later work.

Actually, there are a few investigations carried out by other workers (*3–5*) concerning the effects of hydrolysis of intestinal nuclease and spleen nuclease on the hemolysin-forming potency of pancreatic RNase-pre-hydrolyzed RNA.

In this connection, it may be mentioned that the works on the RNA-splitting enzymes in recent years have led to precise and generally acceptable views on their respective specificities, which circumstances prove very favourable for the excecution of our research.

This paper deals with the effects of phosphodiesterase and phospho-monoesterase hydrolysis on the hemolysin forming potency of yeast RNA and that of pancreatic RNase-predigested RNA.

EXPERIMENTALS

Yeast RNA—This was prepared by Clarke and Schryver's method from brewer's yeast and dialyzed for 3 days against distilled water at pH 7.0 and 4°, which was frequently renewed. The dialyzed solution was lyophilized. This preparation was used as an original sample of RNA. Only some of the experiments on spleen phosphodiesterase digestion were performed with a sample of RNA, in the preparation of which the process of dialysis was omitted from the above-mentioned procedure.

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Pancreatic RNase-digested RNA—RNA prepared by Clarke and Schryver's method was digested by 1/1000 weight of pancreatic RNase (B fraction prepared from beef pancreas according to the method of McDonald (*6*)) for 4 hours at pH 7.4, and dialyzed against distilled water for 24 hours. Then pH of the inside liquid was adjusted to 4.5 and solid NaCl was added to the mixture until a concentration of 1 *M* was obtained. Then cold acetone was added so as to get a concentration of 50 per cent. Letting the reaction mixture stand for 15 minutes at 0°, the resulting precipitate was collected by centrifugation, washed with cold acetone and dried in a vacuum desiccator. The use of crystalline enzyme instead of the above-used enzyme fraction for the digestion of RNA gave no critical difference in the potency of the hydrolysate.

Spleen Phosphodiesterase—The sample obtained from beef spleen according to the method of Heppel *et al.* (*7*) was dialyzed and then lyophilized. This enzyme preparation still contained a small amount of some other enzyme which split the monoester linkage.

Snake Venom Phosphodiesterase—This enzyme was prepared from Habu (*Trimeresurus flavoviridis*) venom by the use of acetone fractionation in the manner analogous to that described by Sinsheimer and Koerner (*8*), and was lyophilized. No appreciable amount of inorganic P was liberated from yeast RNA even after a long period of hydrolysis with this enzyme preparation, and the situation of the phosphomonoester linkage in the digestion product was proved to be at 5′-position of terminal nucleoside by the aid of potato 5′ nucleotidase at pH 9.0.

Potato Phosphodiesterase—5′-nucleotidase fraction prepared by the method of Kornberg and Pricer (*9*) showed a very high phosphodiesterase activity at pH 5.0, thus the preparation was used as phosphodiesterase at this pH. An appreciable liberation of inorganic P was observed during the course of the digestion of RNA by this enzyme preparation at this pH.

RNase of Aspergillus Oryzae—This was prepared from Takadiastase by heat treatment and fractionation with ammonium sulphate*. The preparation, though rather crude, gave no inorganic phosphate in the digestion of RNA.

Prostatic Phosphomonoesterase—As starting material human semen was used. After spermatozoa were removed by centrifugation, the supernatant solution was dialyzed against distilled water for a week at 4°, bacterial growth being precluded by the addition of toluol. The resulting white precipitate was discarded by centrifugation and the clear supernatant was stored at 0°.

All of the above-mentioned enzymes had no or only negligible hemolysin forming activity.

Determination of Potency—This was essentially the same as described in the previous paper (*1-2*). The incubation time to determine the hemolysin-forming potency of RNase-prehydrolysed RNA was fiexed at 30, 60 and 90 minutes, while in the case of yeast RNA hydrolyzed by various enzymes directly, without predigestion with pancreatic RNase, 5, 15, and 30 minute-incubation was chosen.

Enzyme Digestion—The mixture of enzymes and RNA solution (yeast RNA or pancreatic RNase-predigested RNA) was incubated at 37° in suitable buffer solution. After a prescribed time the digested solution was cooled in an ice bath and stored thereafter in a freezing chamber at −5° until the following day when it was lyophilized to determine the potency. To detect the digestion with the enzyme, a part of the digestion mixture was taken out and the acid-soluble phosphorus was measured after the addition of an equal volume of uranyl reagent (2.5 per cent trichloroacetic acid and 0.25 per cent uranyl acetic acid). In two experiments of the spleen phosphodiesterase digestion, digestions were effected in the unbuffered solution and at certain time intervals alkali consumption to establish the initial pH was estimated by the use of 0.1*N* NaOH.

* Further purification of the enzyme has been studied by Sato and Egami (*10*).

In some cases inorganic phosphorus in acid-soluble fraction was measured. Throughout this experiment the phosphorus measurement was conducted following the method of Allen (*11*).

Paper Chromatographic Studies—Descending paper chromatography was carried out with isopropanol-water-ammonia system of Markham and Smith (*12*) for the separation of enzyme-digested products of RNA. Tôyôroshi No. 51 paper was used throughout the studies and the spots on the chromatogram were located by means of ultraviolet light. As the source of ultraviolet light, Matsuda low pressure mercury lamp (steril lamp) with Kaken ultraviolet filter 2537* was employed. Under the illumination of ultraviolet light it is easy to detect the spots on the chromatogram, but, when it was desirous to get the position more definitely, the prints were made by making use of Mitsubishi CH photographic paper.

RESULTS

A) Spleen Phosphodiesterase Digestion—The results of the enzyme digestion of yeast RNA are shown in Tables I, II and Fig. 1.

These data shows that the enzyme digestion of yeast RNA cannot bring

Table I

Potencies of Acetone Fractions of Yeast RNA before and after Spleen Phosphodiesterase Digestion

	Time of digestion	Acetone fractions 0–20% (H.U.)	20–30% (H.U.)	30–50% (H.U.)	50–75% (H.U.)
Before digestion	...	380	230	40	0
After digestion	4	170	100	40	0
	24	...	420	160	

2g. of yeast RNA (containing dialyzable fractions) was digested with 22mg. of the enzyme at 37°. Initial total volume was 35ml. and initial pH was 7.0. During 4-hour incubation the reaction mixture consumed 3.64 ml. of 1/10*N* NaOH so as to regain it initial pH.

Table II

Potencies of Yeast RNA after Spleen Phosphodiesterase Digestion

Time of digestion (*hrs.*)	0	4	8
Potency (H.U.)	95	52	46
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	4.92	87.5	90.5
$\frac{\text{Inorg. P}}{\text{Total P}}$ (%)	0	41.2	45.3

125 mg. of RNA was digested with 80 mg. of the enzyme in 18 ml. of 1/20*M* Tris-buffer at pH 7.0, 37°.

* The authors are indebted to Dr. E. Iwase for his kind gift of this filter.

about such a remarkable enhancement of potency as caused by the digestion with pancreatic RNase.

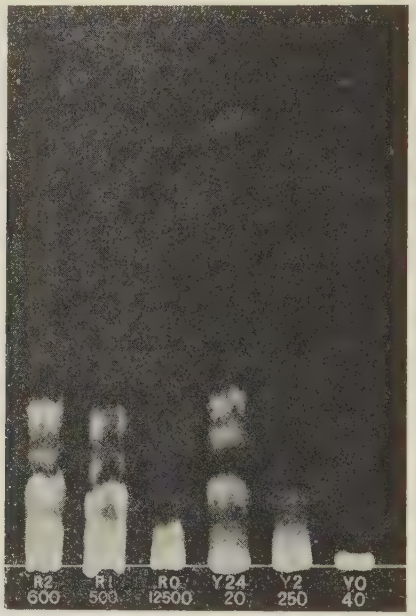

FIG. 1. Chromatogram of the digestion products with spleen phosphodiesterase. Y0, Y2 and Y24 are the digestion products of yeast RNA. 800mg. of yeast RNA was digested with 40 mg. of the enzyme in 50 ml. of distilled water adding NaOH to maintain pH at 7.0 during the digestion. Y0, original RNA; Y2, after 2-hour digestion; Y24, after 24-hour digestion. R0, R1 and R2 are the digestion products of RNase-predigested RNA shown in Table III. R0, original RNase-predigested RNA; R1, after 1-hour digestion; R2, after 2-hour digestion.

TABLE III

Potencies of Pancreatic RNase Predigested RNA after Spleen Phosphodiesterase Digestion

Time of digestion (*hrs.*)	Lyophilized samples after digestion (H.U.)	50% acetone precipitate of dialyze digests (H.U.)
0	12500	
1	5000	17500
2	6000	20000

Each 820mg. of RNase-predigested RNA was digested with 40mg. of the enzyme in 20ml.. of initial total volume of unbuffered solution at 37°, pH 7.0. During 1 and 2-hour incubation 2.95ml. and 4.49 ml. of 1/10*N* NaOH were consumed respectively for the readjustment of pH to the initial value.

TABLE IV

Potencies of RNase-predigested RNA after Spleen Phosphodiesterase Digestion

Time of digestion (*hrs.*)	0	4	8
Potency (H.U.)	29300	16700	9100
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	24.3	95.0	94.3
$\frac{\text{Inorg. P}}{\text{Total P}}$ (%)	0	35.6	67.6

40 mg. of RNase-predigested RNA was digested with 40 mg. of this enzyme in 8 ml. of 1/20*M* Tris-buffer at pH 7.0, 37°.

TABLE V

Potencies of Snake Venom Phosphodiesterase Digests of the Yeast RNA and RNase-predigested RNA

Time of digestion (*hrs.*)	0	2	4	8	16
Yeast RNA					
Potency (H.U.)	20	58	72	94	62
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	0.7	5.4	10.9	21.0	41.4
RNase-predigested RNA Potency	13000	...	12000	12000	9500
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	7.0	...	13.3	18.3	20.9

200mg. of sample was digested with *ca.* 2mg. of the enzyme in 25ml. of Tris-buffer (*M*/20, pH 8.8) in both cases; no inorganic P was found after 18-hour digestion.

Fig. 1 and Tables III and IV shows the results of hydrolysis of RNase-predigested RNA by this enzyme.

These data show that the total potency gradually decreases on account of the hydrolysis by this enzyme, but even in the severely hydrolyzed sample there still remains 1/2 to 1/3 of the initial potency, the potency being kept in larger fragments which cannot be dialyzed.

B) Snake Venom Phosphodiesterase Digestion—Out of several experiments an example is shown in Table V and Fig. 2.

These data show that this enzyme hydrolyzes the RNase-predigested RNA more slowly than the untreated RNA. During the course of hydrolysis of RNA with the enzyme, the rise in hemolysin-forming potency was observed as in the case of alkali hydrolysis, but it was not so remarkable as pancreatic RNase digestion. In the case of RNase-predigested RNA, no appreciable change in hemolysin-forming potency was caused by this enzyme digestion.

C) Digestion with RNase of Aspergillus Oryzae—Table VI shows one of the results of the digestion with this enzyme.

FIG. 2. Chromatogram of digestion products of yeast RNA with snake venom phosphodiesterase indicated in Table V.

TABLE VI

Potencies of Digests of the Yeast RNA and RNase-predigested RNA with RNase of Aspergillus Oryzae

Time of digestion (*hrs.*)	0	4	24
Yeast RNA			
Potency (H.U.)	23	0	0
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	0.5	14.7	37.0
RNase-predigested RNA potency	13000	1900	450
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	3.8	42.5	54.0

160 mg. of yeast RNA was digested with about 0.15 mg. of the enzyme preparation in 25 ml. of 0.2*M* acetate buffer at pH 4.7. 80 mg. of RNase-predigested RNA was digested with about 0.12mg. of the enzyme preparation in 25ml. of 0.2*M* acetate buffer at pH 4.7.

In contrast to the case of other enzyme digestion, considerable decrease in potency was observed even in the case of mild hydrolysis. The situation of hydrolysis was clearly indicated by the chromatographic study (Fig. 3).

D) *Potato Phosphodiesterase Digestion*—With this enzyme only RNase-

FIG. 3. Chromatogram of digestion products with RNase of *Aspergillus oryzae* indicated in Table VI. Ap, yeast adenylic acid; R0, original RNase-predigested RNA; R4, after 4-hour digestion of R0; R24, after 24-hour digestion of R0; Y0, original yeast RNA; Y4, after 4-hour digestion of Y0; Y24, after 24-hour digestion of Y0; RP18, digestion products of R0 after 18-hour digestion with potato phosphodiesterase.

TABLE VII

Potencies of Potato Phosphodiesterase Digests of RNase-predigested RNA

Time of digestion (*hrs.*)	0	2	4	6	21	6*
Potency (H.U.)	13000	6000	3800	4400	4050	8900
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	6.6	38.1	57.8	76.0	84.5	
$\frac{\text{Inorg. P}}{\text{Total P}}$ (%)	0.1	4.2	7.6	10.0	21.3	

250 mg. of pancreatic RNase-predigested RNA was digested by 3.6 mg. of the enzyme preparation 25 ml. in total of 0.1 *M* acetate buffer at pH 5.0.

* Dialyzed sample after 6-hour digestion.

predigested RNA was studied (Table VII).

Where the hydrolysis was further advanced, no appreciable amount of acid insoluble material was found by the addition of uranyl reagent and 23 per cent of total phosphorus was liberated as inorganic phosphorus. The hydrolysate maintained little potency (1100 units) and this potency was not

influenced by the process of dialysis.

This behavior is very similar to that of spleen phosphodiesterase hydrolysis, that is, during the hydrolysis the potency gradually decreased, but even in rather severely hydrolyzed samples 1/3 to 1/2 of their initial potencies were still maintained.

To elucidate these results chromatographic separation was carried out with RNase-digested RNA before and after potato phosphodiesterase digestion (Fig. 4 a and b).

After development with isopropanol-water in ammonia atomosphere, the

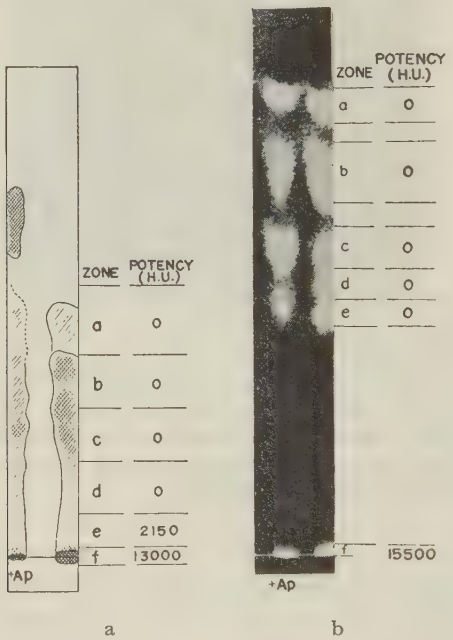

Fig. 4. a. Chromatographic separation of the RNase-digested RNA.
b. Chromatographic separation of the RNase-predigested RNA after the digestion with potato phosphodiesterase for 21 hours.
* A_p: Yeast adenylic acid was added to the sample.

located bands were cut off with the aid of ultraviolet photogram as a guide. The extracts obtained from each strip with distilled water were lyophilized and hemolysin-forming potency was determined in the usual way.

Hemolysin-forming potency was found only at the positions on the starting l ne.

E) Prostatic Phosphomonoesterase Digestion—In the spleen phosphodiesterase and potato phosphodiesterase preparation, considerable amount of phosphomonoesterase activity was found. In order to investigate the effect of the latter on the hemolysin-forming potency, RNase-predigested RNA was hydrolyzed by prostatic phosphomonoesterase (Table VII).

TABLE VIII

Potencies of Prostatic Phosphomonoesterase Digests of RNase-predigested RNA

Time of digestion (*hrs.*)	0	1	2	4	6	21
Potency (H.U.)	13000	9000	13200	9500	14400	5750
$\frac{\text{Soluble P}}{\text{Total P}}$ (%)	11.9	39.2	39.2	41.3	43.2	53.0
$\frac{\text{Inorg. P}}{\text{Total P}}$ (%)	0	19.6	19.6	23.0	25.0	31.7

40 mg. of RNase-predigested RNA was digested with about 430 U. of the enzyme in 25 ml. of 0.1 *M* acetate buffer pH 5.0.

As illustrated in Table VIII, the process of increase of inorganic phosphorus consists of two stages, namely the initial rapidly increasing and later slowly increasing stages. This phenomenon may reasonably be explained by assuming that the initial stage was induced by the direct action of this enzyme on the terminal phosphomonoester linkages originally situated in the substrate, while the later stage was caused by the action on the phosphomonoester linkages secondarily formed by the action of phosphodiesterase which was contained in trace as a contaminant in this enzyme preparation. The hemolysin-forming potencies determined showed some fluctuations in this case, but at least it might well be inferred that a larger part of the terminal phosphomonoester linkages did not play an important role in the hemolysin formation.

DISCUSSION

It has been reported that snake venom phosphodiesterase hydrolyzes phosphodiester linkage in RNA without any specificity for the purine or pyrimidine base of nucleotide and yields 5′-nucleotide (*13*). Then it is naturally conceivable that there is little difference between the amount of purine and that of pyrimidine in the polynucleotide which remains undigested after the treatment with snake venom phosphodiesterase, and that terminal phosphomonoester linkage is situated at 5′-position in the nucleosides. Therefore it is to be presumed that the appreciable rise in potency during the hydrolysis of RNA may only be due to the depolymerization as manifested in alkali hydrolysis.

The failure in detecting any change in hemolysin-forming potency of pancreatic RNase-predigested RNA after the hydrolysis with this enzyme seems to be due to the fact that the structure induced by the action of pancreatic RNase is resistant to this enzyme,

In the spleen phosphodiesterase hydrolysates, it has been found that the RNA hydrolyzed by the enzyme has terminal monophosphate linkage exclusively at 3′-position of terminal nucleoside (*14–16*). This was the reason why we used the enzyme to get some information as to the effect of the structural difference between RNA digested by this enzyme and that of hydrolyzed

by alkali on the hemolysin-forming potency. However, we could not find critical difference in the effect on the potency between these two as described in the foregoing section. Therefore, it seems unreasonable to assume the inhibiting action of 2′-monophosphate linkage which might be formed in the alkali hydrolysis.

As reported also by Heppel (*3*), the active structure of pancreatic RNase-predigested RNA was relatively resistant to the hydrolysis with this enzyme. This fact is similar to the case of the potato phosphodiesterase digestion of the RNase-predigested RNA. In both cases dialyzed samples have large potency than before dialysis. Therefore, the active substance seems not so small as to be dialyzable. This is confirmed by the fact that the sample which is completely digested by potato phosphodiesterase and therefore gives no precipitate with uranyl acetate has only a little potency.

The chromatographic study could elucidate this situation very clearly. Furthermore, the results of monoesterase digestion showed that terminal phosphomonoester linkage played no appreciable part in hemolysin-forming potency.

These findings lead us to the following considerations:

1. The formation of active substance depends upon the specificity of pancreatic RNase.
2. This substance has some resistance to the above-mentioned three phosphodiesterase, and intestinal phosphodiesterase.
3. Terminal phosphomonoester bonds plays little role in the potency.
4. The active substance seems to be a fairly large oligonucleotide, acid insoluble and nondialyzable, although the possibility that the presence of inhibiting substance in small fragments may diminish the activity of the fraction which contains them can not be excluded.

Digestions of the yeast RNA and pancreatic RNase-predigested RNA with RNase of *Aspergillus oryzae* provoked rapid loss of hemolysin-forming activity. Further studies on the specificity of this enzyme would prove some aid for elucidating the structure of the active substance.

Further investigation is in progress in our laboratories with a view to clarifying the structure of the substance.

SUMMARY

1. Spleen phosphodiesterase digestion of yeast RNA showed no critical change in its potency. The finding by Heppel that the active structure in pancreatic RNase-predigested RNA is resistant to this enzyme was confirmed.

2. Considerable rise in hemolysin-forming potency, such as shown in alkali hydrolysis, was detected during the hydrolysis of yeast RNA by Habu-venom phosphodiesterase. Pancreatic RNase-digested RNA was scarcely affected by this enzyme digestion.

3. Potato phosphodiesterase digestion manifested the effect similar to that of spleen phosphodiesterase digestion on the RNase-predigested RNA.

Hydrolysates were separated by means of paper chromatography with isopropanol-water-ammonia system and potency was found only as the starting position.

4. During digestion with RNase of *Aspergillus oryzae*, unlike other phosphodiesterase, potency of the yeast RNA and pancreatic RNase-digested RNA diminished very rapidly.

5. Prostatic phosphomonoesterase digestion had no critical effect on the potency of RNA.

6. Some discussions were given as to the possible structure of the active substance.

The authors are indebted to Prof. F. Egami for the interest he has taken and the encouragement he has given during this work.

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BIOCHEMICAL STUDIES ON STREPTOLYSIN S.

IV. FRACTIONATION OF ACTIVE SUBSTANCE FOR STREPTOLYSIN FORMATION AND ITS PROPERTIES*

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As already reported in the preceding papers of this series (*2–4*), a remarkable increase in streptolysin-forming activity was observed when yeast ribonucleic acid (RNA) was digested with pancreatic ribonuclease (RNase). No such remarkable effect was obtained by the action of alkali or several other enzymes toward yeast RNA. The fact and the nondialysablility of the active substance indicated that it is rather large molecular oligonucleotides formed by the specificity of the pancreatic RNase.

In the present study, the fractionation of the RNase-resistant material by means of paper chromatography, dialysis, electrophoresis and ECTEOLA-cellulose column chromatography was carried out and the streptolysin-forming activity of the resultant fractions was determined. Furthermore, the effect of the terminal pyrimidine nucleotide on the activity was also investigated. The results showed that the active substance is of rather large molecular weight and has a high content of guanylic acid and that the terminal pyrimidine nucleotide has little effect for the streptolysin-forming activity.

EXPERIMENTAL

Yeast Ribonucleic Acid (*YNA*)—The following three preparations of YNA were used.

YNA-1: Commercial yeast RNA (sodium salt, Merck; P, 8.1 per cent).

YNA-2: Commercial yeast RNA (sodium salt, Schwarz; P, 8.3 per cent).

YNA-3: YNA-2 was deproteinized with chloroform gel formation, dialyzed in a cellophane tubing against distilled water, and then lyophilized. (sodium salt: P, 8.2 per cent).

RNase-resistant Material of YNA (*RM*)—The following samples were used.

RM-1: 10 g. of YNA-1 were digested with 12 mg. of crystalline RNase in 200 ml. of Tris buffer (pH 7.4) at 37° for 4 hours. The digested mixture was dialyzed against distilled water exhaustively and then lyophilized. The lyophilized material was dissolved in 1.0 *M* NaCl solution, pH of the solution was adjusted to 4.5 and then cooled to 0°. To the solution, cold acetone was added up to 30 per cent concentration, thereafter the precipitate was collected by centrifugation, washed with acetone three times, and then dried in a vaccum desiccator.

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RM-2A: 50 g. of YNA-2 were deproteinized with chloroform gel formation and dialyzed exhaustively against distilled water. Dialyzed material (in 250 ml.) was digested with 8.5 mg. of the crystalline enzyme for 18 hours at 37° with frequent addition of NaOH to maintain the pH at 7.2. Digested solution was throughly dialyzed in a cellophane tubing against distilled water. (sodium salt: P, 8.3 per cent)

RM-2B: RM-2A was dissolved in distilled water, dialyzed in a colodium bag against distilled water, and then lyophilized. (sodium salt: P, 8.1 per cent)

RM-3: 15 g. of YNA-3 were dissolved in 150 ml. of distilled water and digested with 15 mg. of crystalline RNase for 40 hours as in the case of RM-2A. Chloroform was added to prevent bacterial growth. The digested solution was exhaustively dialyzed in a cellophane tubing against distilled water.

Alkali-resistant Material—5 g. of YNA-3 were dissolved in 25 ml. of distilled water (pH 7.0) and cooled. To the cold solution kept in an ice bath, 25 ml. of prechilled 2 *N* NaOH were added carefully and the mixture was allowed to stand 0° for 3 hours. The solution was neutralized with 5 *N* HCl under cooling, and then exhaustively dialyzed in a cellophane tubing against distilled water.

ECTEOLA-cellulose—This was prepared by the method of Peterson and Sober (*5*) from Carl Schlicher and Schüll cellulose powder No. 123. Nitrogen content of the sample was 0.39 per cent (0.28 meq./g.).

Prostatic Phosphomonoesterase—The enzyme used in the study was prepared from human prostate tissue by the method of Schmidt (*6*). 1 ml. of the enzyme solution contained about 800 units of activity. (*7*).

Paper Chromatographic Separation—For the separation of degradation products of RNA, descending paper chromatography was carried out using isopropanol-water-ammonia as the solvent system according to Markham and Smith (*8*). The spots on chromatograms were located by ultraviolet photography, as described in the preceding paper of this series (*4*).

Dialysis of the RNase-resistant Material—100 mg. of RM-2B were dissolved in 2 ml. of 2 *M* NaCl and dialyzed in a cellophane tubing against 8 ml. of 2 *M* NaCl solution. Outer liquid of the dialysis was changed three times with 10 ml. of 2 *M* NaCl solution after each 24 hours. During the dialysis a small amount of precipitate was formed in the tubing. After 4 day dialysis, the precipitate in the tubing was separated from the supernatant by centrifugation, washed with 1 ml. of 2 *M* NaCl, then dissolved in distilled water, and dialyzed against distilled water. The outer liquid and the suparnatant in the tubing were also dialyzed against distilled water under mixing. All of the dialyzed samples were finally lyophilized.

Electrophoretic Fractionation—Zone electrophoresis was carried out with potato starch as supporting medium in an apparatus simillar to that of Kunkel and Slater (*9*). The buffer used was 0.05 *M* ammonium formate at pH 3.5 or 0.1 *M* phosphate at pH 7.0. The sample was mixed with starch and placed in a 1 cm. slit which was cut about 15 cm. from the cathode end. The whole starch block was 40×5×1 cm. in size. At the end of run, the starch block was blotted by a filter paper strip and the approximate positions of oligonucleotides were estimated by observation of the strip under ultraviolet light after drying. Then the starch block was cut into 1 cm. sections, and each section was eluted with distilled water. Oligonucleotides in the eluates were estimated by measuring optical density at 260 mμ.

Fractionation on ECTEOLA-cellulose Column—The RNase-resistant material of yeast RNA was adsorbed on the column from the 0.01 *M* neutral phosphate buffer and eluted with sodium chloride solution. The salt concentration was discontinuously increased as reported for the case of RNA and DNA (*10–11*). More details of the method will be described elsewhere.

Removal of the Terminal Pyrinidine Nucleotide—300 mg. of RM-3 were treated with 10 ml. of 0.1 *N* HCl for 4 hours at 20–25° to cleave the cyclic phosphate bonds which might be present in the RNase digestion product. The hydrolyzate was neutralized with 0.5 *M* sodium acetate

to pH 5.4 and diluted to 25 ml. To 5 ml. of the solution, 720 units of prostatic phosphomonoesterase dissolved in 4.5 ml. of distilled water was added and incubated for 2 hours. The enzyme action was stopped by heating the mixture at 65° for 5 minutes as pH 7. The solution was then acidified with 1/9 volume of *N* HCl. The resultant precipitate was collected by centrifugation, and washed with 50 per cent aqueous acetone and then with 100 per cent acetone. The washed precipitate was dried *in vacuo*. To confirm the removal of the terminal phosphate, a portion of the precipitate was treated with prostatic phosphomonoesterase once more. No inorganic phosphorous was, however, formed in this case. 10 mg. of the precipitate were dissolved in 1.3 ml. of distilled water (pH 6.4), 1.3 ml. of 0.22 *M* $NaIO_4$ were added and kept at room temperature for 30 minutes. The mixture was then diluted to *ca.* 10 ml. and dialyzed overnight against distilled water in a refrigerator, a few drops of chloroform being added to prevent bacterial contamination. The dialyzed solution was then lyophylized. The dried material was dissolved in glycine buffer of pH 10 and incubated at 37° with the addition of a few drops of chloroform for 24 hours. The mixture was neutralized with 0.1 *N* HCl and lyophilized. The lyophilized material was chromatographed using isopropanol-water-ammonia as developing system. The band located near the starting line of the chromatogram was cut off extracted with distilled water, and lyophilized.

Determination of Molar Ratio of the Base—Analyses for base of RNA and its degradated products were carried out by paper chromatography according to the method of Smith and Markham (*12*). A mixture of *tert.*-butanol and HCl was used as a solvent. Tôyô 51A filter paper was used throughout the analyses.

Determination of Potency—This was carried out in the same way as described in the preceding papers. (*2–4*).

RESULTS

Paper Chromatographic Separation—As shown in the preceding paper (*4*), it was indicated that a highly active substance present in the RNase digest of yeast RNA is found near the starting line of the chromatogram, even after digestion with potato phosphodiesterase.

In order to obtain further information on the properties of the active substance, chromatographic behavior of samples described below was investigated.

Yeast RNA was partially hydrolyzed with alkali (1 *N* NaOH, 30 minutes 0°) and fractionated between 30 and 50 per cent acetone concentrations (see (*4*)). This fraction was further digested with RNase for 27 hours and examined by paper chromatography. The chromatogram thus obtained is shown in Fig. 1.

In Fig. 2 is shown the result of chromatography of the fraction precipitated between 20 and 30 per cent acetone concentrations from RNase digest (18 hours) of yeast RNA (see (*4*)).

Even in the case of the highly potent fraction (Fig. 2), the active substance could be found only in the vicinity of the starting line. Furthermore, the potencies of the eluates from the starting positions of these chromatograms differed.

From the comparison of base ratio of the original substance (RNase-resistant material) and that of the active fraction eluted from the chromatogram, it was concluded that the active fraction is rich in guanylic acid and

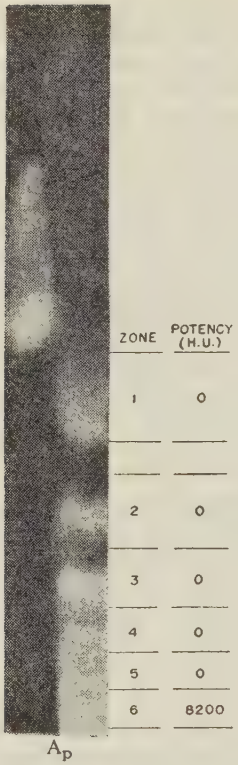

FIG. 1. Chromatogram of the alkali-predigested RNA after 27 hours RNase digestion. Yeast RNA was partially hydrolyzed with alkali (1 *N* NaOH, 0°, 30 minutes) and fractionated between 30 and 50 per cent acetone concentrations. This fraction was further digested with RNase 27 hours. Potency of starting substance: 5500 H.U. Ap: yeast adenylic acid.

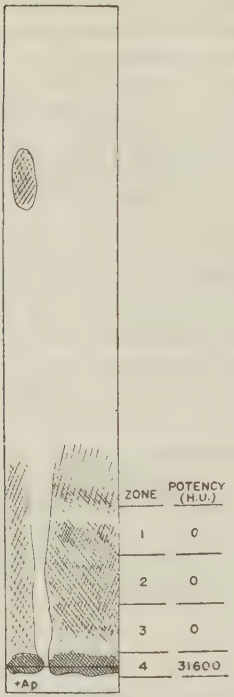

FIG. 2. Chromatogram of the fraction precipitated between 20 and 30 per cent acetone concentrations from RNase digest (18 hours) of yeast RNA. Potency of starting substance: 28000 H.U. Ap: yeast adenylic acid was added to the sample.

TABLE I

*Base Ratios of the RNase Digest of Yeast RNA and Its Active Fraction Eluted from the Paper Chromatogram**

Sample	Base ratio** A	G	U	C	Pu/Py
Spotted RNase digest	10	14.5	3.0	3.3	3.9
Eluted active fraction	10	30.0	3.7	3.6	5.5

* Ref. Fig. 2.

** A: adenine, G: guanine, U: uracil, C: cytosine.

contains rather large number of purine base per one terminal pyrimidine.

Fractionation by Dialysis—In a previous paper (*2*), it was shown that the active substance of RNase-resistant material is found predominantly in the nondialyzable material by the dialysis in a cellophane tubing against distilled water. It was shown by Markham and Smith (*13*) that the dialyzability of the RNase-resistant material is greatly increased if the water outside the dialysing tubing is replaced by 2 *M* NaCl.

To obtain information concerning the nature of active substance, the RNase-resistant material (RM-2A) was dialyzed in a colodium bag exhaustively and lyophilized (RM-2B). Furthermore, the RM-2B was dialyzed again against 2 *M* NaCl and potencies and base ratios of the resultant fractions were compared.

TABLE II

Potencies and Base Ratios of the Fractions Obtained by Dialysis of RNase-resistant Material

Sample	Base ratio A	G	U	C	Pu/Py	Potency (H.U.)
RM-2A	10	14.2	6.1	6.7	1.9	22000
Dialysis of RM-2A in a colodium bag						
Dialyzable material	—	—	—	—	—	290
Nondialyzable material (RM-2B)	10	17.8	4.2	4.4	3.2	57000
Dialysis of RM-2B against 2 *M* NaCl						
Dialyzable material	10	8.0	4.3	2.9	2.5	—
Nondialyzable material						
Suparnatant	10	24.9	3.9	4.1	4.4	73600
Precipitate	10	19.3	2.8	3.2	4.9	26500

RM-2A was dialyzed in a collodium bag against distilled water and lyophilized (RM-2B).

RM-2B was dialyzed in a collophane tubing against 2 *M* NaCl. The precipitate formed in the tubing during the dialysis, the suparnatant remained in the tubing, and outer liquid collected were dialyzed against distilled water under rapid staring. All of the samples were finally lyophilized.

As shown in Table II, the active substance remains in the dialysing tubing even by the dialysis against 2 *M* NaCl. This fact and its base ratio also suggest that the active substance is larger molecular substance containing high content of guanylic acid.

Electrophoretic Separation—Zone electrophoresis was applied to separate a large amount of the RNase-resistant material.

Fig. 3 shows the result obtained with RM-1 at pH 7.0. The electrophoretic pattern obtained by measuring optical density at 260 mμ possessed only one peak for the oligonucleotide distribution, but the activity was associated with fractions of larger mobility.

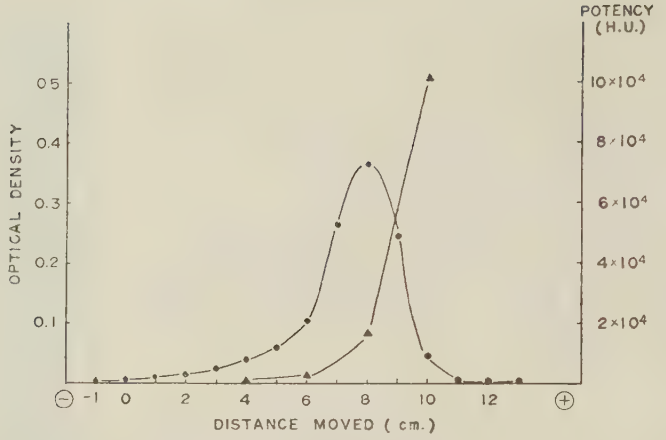

Fig. 3. Starch electrophoresis pattern of RNase-resistant meterial (RM-1). Phosphate pH 7.0, 4.5 hours, 6.5 volts/cm. Each section was eluted with distilled water (total 5 ml.). Optical density was measured after 400-fold dilution. ●——● shows distribution of oligonucleotides (readings of O.D. at 260 mμ). ▲——▲ shows streptolysin forming potency of lyophilized materials obtained from each section.

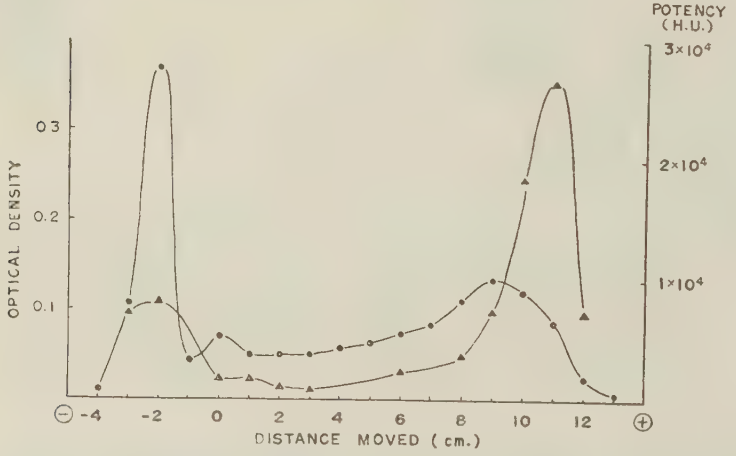

Fig. 4. Starch electrophoresis pattern of RNase-resistant material (RM-1). Formate pH 3.5, 9.5 hours, 10 volts/cm. Each section was eluted with distilled water (total 10 ml.). Optical density was measured after 200-fold dilution. ●——● shows distribution of oligonucleotides (readings of O.D. at 260 mμ). ▲——▲ shows streptolysin forming potency of lyophilized materials obtained from each section.

Fig. 4 shows the pattern obtained in electrophoresis at pH 3.5. In this case, a sharp peak near the starting position together with a broad peak was observed for the oligonucleotide distribution. The active substance, however,

TABLE III

*Base Ratios of the RNase Digest of Yeast RNA and Electrophoreticaly Fractionated Materials**

Sample	Base ratio A	G	U	C	Pu/Py
RM-1	10	15.0	3.7	4.1	3.2
Fraction 11**	10	28.1	4.7	4.3	4.2
Fraction 6**	10	12.9	3.7	2.8	3.5
Fraction -2**	10	15.5	3.0	4.2	3.5

* Ref. Fig. 4.

** Number of the fraction presents the distance from the starting position (cm.).

was found predominantly in the anodic front of the pattern. The base ratio of the active fraction showed a high content in guanylic acid as in the case of paper chromatographic separation.

ECTEOLA-cellulose Column Chromatographic Fractionation—The ionic property of the active substance revealed in the electrophoretic separation was more successfully utilized in ECTEOLA-cellulose column chromatography.

The active substance was eluted from the column with NaCl solution, concentration of which was more than 0.3 *M*. A very sharp resolution was

TABLE IV

*Molar Base Ratios and Activity of Fractions Obtained by ECTEOLA-cellulose Column Chromatography**

Sample	Base ratio A	G	U	C	Pu/Py	Potency (H.U.)
RM-2B	10	17.8	4.2	4.4	3.2	57000
Fraction eluted with						
0.1 *M* NaCl	10	11.7	5.1	5.9	2.0	215
0.2 *M* NaCl	10	12.3	2.8	3.6	3.5	9700
After rechromatography						
0.2 *M* NaCl	10	9.8	2.5	2.4	4.0	—**
0.3 *M* NaCl						19200***
0.3 *M* NaCl	10	30.2	3.5	4.2	5.2	45600
After rechromatography						
0.3 *M* NaCl	10	30.2	5.0	3.6	4.7	59200
0.4 *M* NaCl	10	31.8	4.0	3.5	5.6	91000
0.5 *M* NaCl	10	31.8	4.9	3.4	5.0	80800

* Ref. Fig. 5A and 5B.

** Inactive in the cencentration of 20 μg./ml.

*** So small amount was obtained that the base ratio could not be measured.

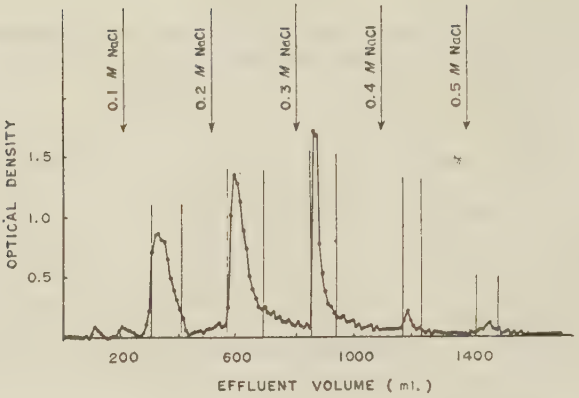

Fig. 5A. Stepwise elution diagram of RNase-resistant material (RM-2B) from a column of ECTEOLA-cellulose with NaCl solution (in 0.01 *M* phosphate). Exchanger: 20 g. ECTEOLA-cellulose, 2.8 cm. in diameter. Sorbed material: 300 mg. of RM-2B. The optical density (at 260 mμ) was measured after 20 fold dilution of the eluate in 1 cm. cells. About 90 per cent of U.V. absorbing substance was recovered. The fractions indicated with the mark | | were used for the determination of potency and base radio.

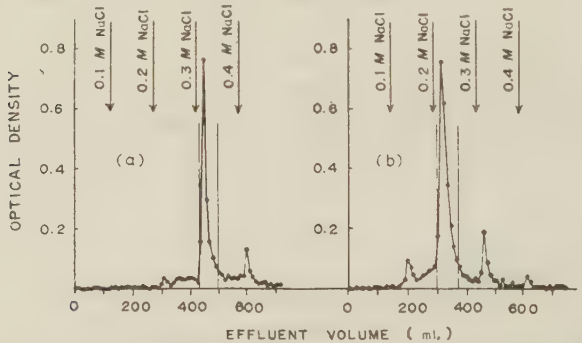

Fig. 5B. Rechromatography of the fractions obtained from the peaks of 0.3 *M* (a) and 0.2 *M* (b) NaCl respectively, as shown in Fig. 5A (Each *ca.* 33 mg.). Column size: 10 g. of ECTEOLA-cellulose, 1.8 cm. in diameter. The optical density (at 260 mμ) was measured after 20 fold dilution of the eluate in a 1 cm. cell. The fractions indicated with the mark | | were used for the determination of potency and base ratio.

obtained as shown in Fig. 5 and Table IV. Furthermore, an excellent parallelism was observed between the guanylic acid content and the activity. From the determination of the ratios of purine to pyrimidine of these fractions and the specificity of pancreatic RNase, it was assumed that the active substance possesses more than five or six nucleotides.

As might be suggested from the results of alkaline degradation in a

preenhancement of the activity, observed in RNase digests of YNA, was due to the specificity of pancreatic RNase. The results of the fractionation of the vious paper (*2*), only a little activity was found in fractions obtained by ECTEOLA-cellulose column chromatography of alkali-resistant material. (Fig. 6, Table V).

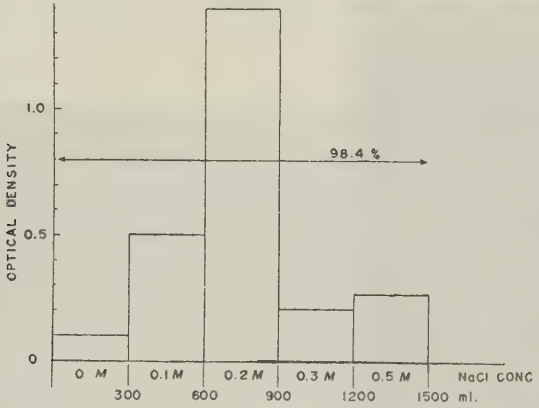

FIG. 6. Stepwise elution diagram of alkali-resistant material from a column of ECTEOLA-cellulose with NaCl solution (in 0.01 *M* neutral phosphate). Exchanger: 20 g. of ECTEOLA-cellulose, 2.8 cm. in diameter. Sorbed material: 300 mg. of alkali-resistant material.

TABLE V

*Molar Base Ratios and Activities of the Fractions Obtained by ECTEOLA-cellulose Column Chromatography of Alkali-resistant Material of Yeast RNA**

Sample	Base ratio A	G	U	C	Potency (H.U.)
Original alkali-resistant fraction	10	12.3	9.3	9.0	55
Fraction eluted with					
0.1 *M* NaCl	10	12.3	12.4	11.3	—**
0.2 *M* NaCl	10	12.4	8.3	9.2	31
0.3 *M* NaCl	10	12.3	7.3	7.9	36
0.5 *M* NaCl	10	12.5	7.1	8.3	245

* Ref. Fig. 6.

** Inactive in the concentration of 1 mg./ml.

No definite differences in base ratio were observed among the fractions.

Removal of the Terminal Pyrimidine Nucleotide—From the specificity of pancreatic RNase, it might be easily expected that the pyrimidine nucleotide locates at the terminal position of the active oligonucleotide. Then the effect of the terminal pyrimidine nucleotide on the streptolysin-forming activity was

investigated. An attempt was, therefore, made to eliminate the terminal nucleotide by a method similar to that of Whitfeld for the stepwise degradation of polynucleotides (*14*). The method consisted of four steps, namely treatment with 0.1 *N* HCl, phosphomonoesterase digestion, $NaIO_4$ oxidation and treatment with weak alkali. After the final step, the sample

FIG. 7. Chromatogram of the RNase-resistant material obtained after the treatment to remove its terminal pyrimidine nucleotide. *Ap: yeast adenylic acid, A: sample obtained after $NaIO_4$ treatment, B: sample obtained after final step.

TABLE VI

*Potencies of the RM-3 (RNase Resistant Material) after the Treatment to Remove Its Terminal Pyrimidine Nucleotide**

Sample	No. of experiment	Potency (H.U.)
RM-3		30000
After monoesterase digestion		19300
After final step	1**	30000
	2	27000

* Ref. Fig. 7.

** The substance was eluted from the band 1 of the chromatogram shown in Fig. 7.

was lyophilized and examined by paper chromatography (Fig. 7). Then the band located near the starting line was eluted with distilled water and then lyophilized. The lyophilized sample was divided into two portions, and one was used for the determination of potency and the other for the detection of bases. The potency of the phosphomonoesterase digest obtained above was also measured (Table VI).

Although little pyrimidine nucleotide was detected by base analysis of the final product (Fig. 8), no remarkable effect on the potency was caused by the above treatments.

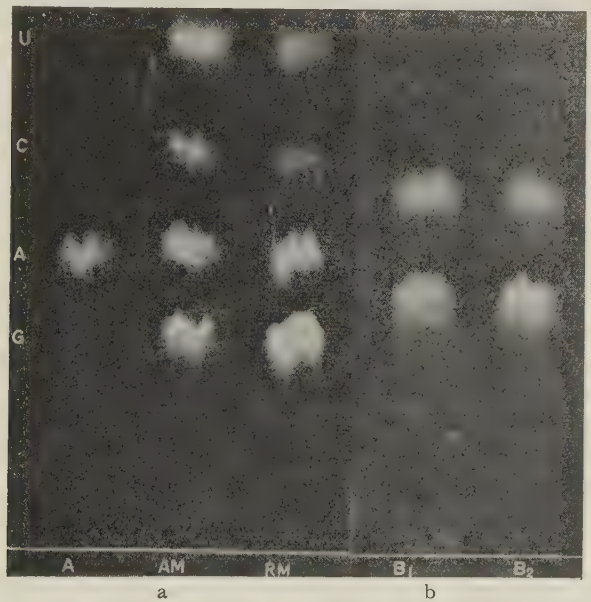

FIG. 8. Chromatographic detection of the bases. Each sample was hydrolyzed with 1 *N* HCl for 1 hours at 100° and chromatographed using *tert.*-butanol-HCl-H_2O as a solvent. A: yeast adenylic acid, AM: alkali-resistant material, RM: RNase-resistant material, B_1: the sample obtained from band 1 of Fig. 7, B_2: the sample obtained from band 2 of Fig. 7. A: adenine, G: guanine, C: cytidylic acid, U: uridylic acid.

DISCUSSION

It has been shown in the preceding papers (*2–4*) that the remarkable RNase-resistant material with four methods, as shown above, clearly indicated that the active substance is rich in guanylic acid.

It has also been shown by Bernheimer (*15*) that RNA prepared from tobacco mosaic virus failed to exhibit such effect as caused by yeast RNA. On the other hand, RNAs prepared from wheat germ, mammalian liver, tobacco leaves, and streptococci and their RNase digests showed nearly the same activity as in the case of yeast RNA.

Berinheimer's interesting results on the tobacco mosaic virus RNA were recently confirmed in this laboratory,* and it was indicated that tobacco mosaic virus RNA and its RNase digest were poor in guanylic acid. The fact reflects the important role of the guanylic acid in the active substance.

Furthermore, this conclusion was also supported by an investigation with enzymatically synthesized polynucleotides (*16*). It has been shown that the enzymatically synthesized polynucleotide containing relatively large amounts of guanylic acid was more active than yeast RNA, while guanylic acid-poor polynucleotide showed only a little potency even after the RNase digestion.

In the preceding paper (*4*), it has been shown that the activity of the RNase-digested yeast RNA was rapidly decreased by the action of RNase prepared from *Aspergillus oryzae.*** Further studies on the purification and specificity of the enzyme by Sato and Egami (*17*) showed the existence of a specific RNase (T_1) which predominantly splits the internucleotide bond adjacent to guanylic acid residue. The fact is also consistent with the view that the active substance contains a large amount of guanylic acid.

The purine to pyrimidine ratio of the active fraction eluted from the ECTEOLA-cellulose column also suggested that the active substance is composed of more than five or six mononucleotides.

The elucidation of the sequence of such a large molecular oligonucleotide may have to await further progress of the method of purification. At least, however, the terminal pyrimidine nucleotide does not seem to be essential for the activity, as shown in this study.

From these facts, it seems likely to consider that most of the streptolysin-forming activity is induced by polyguanylic acid structures composed of more than three or four guanylic acid residues.

SUMMARY

The active substance for streptolysin formation in pancreatic RNase digests of yeast RNA was fractionated with paper chromatography, dialysis, electrophoresis and ECTEOLA-cellulose column chromatography. The results showed that the active substance is very rich in guanylic acid residue and composed of more than five or six mononucleotides. Removal of the terminal pyrimidine nucleotide has no remarkable effect on the potency of the active substance.

The author wishes to express appreciation to Prof. F. Egami for his helpful suggestions and encouragement. He is also indebted to Dr. T. Hayashi and Miss S. Maekawa for the determination of potency.

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STUDIES ON AMYLASE FORMATION BY BACILLUS BUBTILIS

IV. THE RELATIONSHIP BETWEEN NUCLEIC ACID SYNTHESIS AND AMYLASE FORMATION*

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It has been postulated by a number of workers that ribonucleic acid plays an important role in protein synthesis, but the detailed mechanism of its action is still unkown. The first question which poses itself in this connection is whether the continued synthesis of new RNA molecule is required for enzyme formation or the mere presence of RNA is suffieient for its catalytic role in the enzyme formation. From the study of β-galactosidase formation by pyrimidine requiring strain of *Escherichia coli*, Pardee concluded that continuous formation of RNA is essential for protein formation (*1*). Similar conclusion has been drawn by Spiegelman *et al.* (*2*). On the other hand, Creaser has suggested, from his experiment using 8-azaguanine as a purine analogue, that the synthesis of RNA is essential for the formation of inducible enzyme but not for the formation of constitutive enzyme (*3*).

It was reported that the high molecular weight substance isolated from the boiled cell extract had an activating effect on the formation of amylase both by lysozyme lysed cell preparation and by intact cells (*4,5*). The nature of this substance was studied and it was established to be RNA (*5, 6*). Along with the line of this approach, the investigation was undertaken to examine the relationship between the synthesis of RNA and the formation of amylase, using 8-azaguanine and 2,4-dinitrophenol as inhibitors of nucleic acid metabolism. The results obtained were presented and discussed in this paper.

METHODS

Bacillus subtilis H, which has been used throughout the previous studies, was used in this investigation.

The composition of LBY medium is as follows: lactose 1 per cent, peptone 1 per cent, meat extract 0.5 per cent, yeast extract 0.2 per cent, NaCl 0.2per cent, $Na_2HPO_4 \cdot 12H_2O$ *M*/30, pH=7.2: LBY (E, 7.5) medium ; LBY medium fortified with 7.5 per cent polyethylene glycol (molecular weight, 400) : ZLE medium: lactose 1 per cent, peptone 0.05 per cent, monosodium glutamate 0.01 *M*, sodium citrate 0.02 *M*, $(NH_4)_2HPO_4$ *M*/15, $MgSO_4 \cdot 7H_2O$ 0.002 *M*, polyethylene glycol (molecular weight, 400) 10 per cent, pH=7.2. Amylase was

* The following abbreviations will be used: 8-AG, 8-azaguanine; DNP, 2,4-dinitrophenol; RNA, ribonucleic acid; RNAase, ribonuclease.

assayed by the method of Hagihara (*7*). Nucleic acid and protein fractions were extracted by the modified method of Schneider (*8*). Radioactivity was determined by the conventional Ceiger-Müller counter with an end window.

Uniformly C^{14}-labeled adenylic acid was prepared from the nucleic acid fraction of *Euglena* grown in the presence of $C^{14}O_2$ as a sole source of carbon (*9*). The preparation contains adenosine 2′-and 3′-phosphates.

RESULTS

Effects of 8-AG and DNP on the Formation of Amylase at Various Stages of Cultural Development—As shown in the previous paper (*10*) the phase of active amylase formation of this bacterium differs from that of cell multiplication. The growth and amylase formation in ZLE medium were shown in Fig. 1.

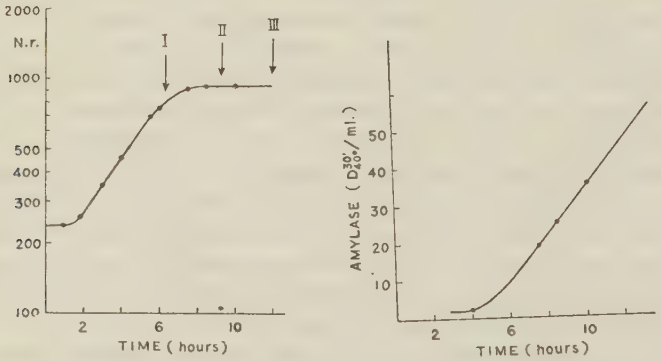

Fig. 1. Growth curve and amylase formation in ZLE medium. Cells were grown in LBY medium for 18 hours. One volume of this culture was added to 10 volume of fresh ZLE medium (time 0) and further cultivated under aeration. Growth and amylase formation were then followed.

It was supposed that the amylase-forming system might be synthesized only at some critical stage, probably the transitory stage from the phase of cell multiplication to that of active amylase production. Therefore, the relationship between the synthesis of nucleic acid and the formation of amylase was examined at various stages of cultural development using 8-AG and DNP as inhibitors of nucleic acid metabolism. Both inhibitors were used at the concentrations where they were demonstrated to be effective in inhiliting the cellular growth and, consequently, the subsequent amylase formation when added in the log phase (Fig. 2).

Cells were grown in ZLE medium at 30°. At various phases indicated in Fig. 1, the inhibitors were added to the culture and their effects on the amylase formation were examined. Results are presented in Table I.

While DNP showed little inhibition on the formation of amylase at either phase tested, 8-AG exerted a pronounced inhibitory effect at phase I, that is, at the early phase of amylase production. The inhibition by 8-AG was not recognized at later stages. The fact that amylase forming system is sensitive

to 8-AG only at some critical stage (phase I) seems to suggest that nucleic acid component of amylase forming system is synthesized at this critical stage. However, when cells at later phases (phase II and III) were separated from the medium and resuspended in new ZLE medium, a considerable inhibition was often observed. Therefore, this interesting possibility was not supported, though not excluded.

Reversal of 8-AG Inhibition by Guanine and Yeast RNA—From the experimental results presented in Fig. 3, it was shown that 8-AG inhibition could be reversed by the addition of either guanine ($1.1\times10^{-4}M$) or yeast RNA ($4.4\times10^{-3}M$) to the medium. Therefore, it is clear that the inhibitory effect of

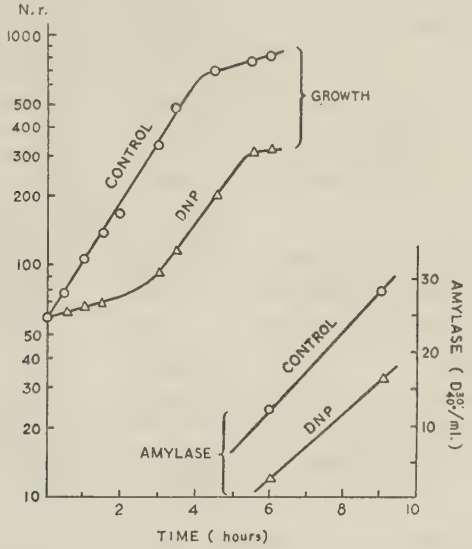

Fig. 2. Effect of 2,4-dinitrophenol (DNP) on the cellular growth and amylase formation of the log phase culture. Cells were grown in LBY medium for 16 hours, at 30°. 1 volume of this culture was added to 10 volume of fresh LBY (E, 7.5) medium with or without DNP (final concentration $0.5\times10^{-4}M$) (time 0), and further aerated. Growth and amylase formation were then followed.

8-AG on the enzyme formation relates specifically to the nucleic acid metabolism. The apparent absence of 8-AG inhibition in the later phase as described in the previous section was interpreted to be due to the accumulation of the degradation products of nucleic acid in the medium or within the cell.

Effect of DNP on the Incorporation of P^{32}-Phosphate and C^{14}-Adenylic Acid into the Nucleic Acid Fraction—As described in the previous section, DNP at the concentration of 0.5 to $1.5\times10^{-4}M$ did not inhibit the enzyme formation when added after cell growth had ceased. At the same concentration, DNP could effectively inhibit cell growth and subsequent amylase formation if

added during the log phase (Fig. 2). Therefore, DNP seems to act as an effective uncoupler at this concentration, and presumably, to derange the

TABLE I

Effects of 8-AG and DNP on the Amylase Formation at Various Growth Phases

Exptl. No.	Growth phase*	Addition (final conc.)	Amylase ($D_{40°}^{30'}$/*ml.*)				
			Initial	Increase		Inhibition (%)	
				2 hr.	3 hr.	2 hr.	3 hr.
1	I	—	7.3		15.2		—
		8-AG (5×10^{-3} *M*)	7.3		4.3		72
	II	—	23		18		—
		8-AG (5×10^{-3} *M*)	23		20		(−10)
	III	—	40		15		—
		8-AG (5×10^{-3} *M*)	40		15		0
2	I	—	5.6		13.2		—
		DNP (0.5×10^{-4} *M*)	5.6		13.2		0
		DNP (1.5×10^{-4} *M*)	5.6		13.8		(−5)
		8-AG (5×10^{-3} *M*)	5.6		6.2		53
	II	—	16		17.6		—
		DNP (0.5×10^{-4} *M*)	16		14.6		17
		DNP (1.5×10^{-4} *M*)	16		14.6		17
		8-AG (5×10^{-3} *M*)	16		14.6		17
	III**	—	1.0	7.1	11	—	—
		DNP (0.5×10^{-4} *M*)	1.0	7.1	10.6	0	4
		DNP (1.5×10^{-4} *M*)	1.0	7.1	9.9	0	9
		8-AG (5×10^{-3} *M*)	1.0	8.0	11.4	(−11)	(−14)

* Cells were grown in LBY medium for 18 hours. One volume of this culture was added to 10 volume of fresh ZLE medium and further cultivated with shaking. At the growth phase I, II and III indicated in Fig. 1, 5 ml. of culture was withdrawn, added to 1 ml. of each of the various inhibitor solution as indicated in the table (time 0). Amylase was assayed after 2 or 3 hours of incubation.

** In this experiment, cells were separated from the culture medium, resuspended in the same volume of fresh ZLE medium as that of original culture. 5 ml. of this cell suspension was added to 1 ml. of each of the various inhibitor solutions and experiment was performed in the same way as in other experimets.

nucleic acid metabolism. The question now arises, whether amylase formation can really take place under the condition where nucleic acid synthesis is

severely damaged. Isotopic experiments were undertaken to answer this question.

The incorporation of P^{32}-phosphate and C^{14}-adenylic acid into nucleic acid fraction was used as a measure of nucleic acid synthesis. Labeled com

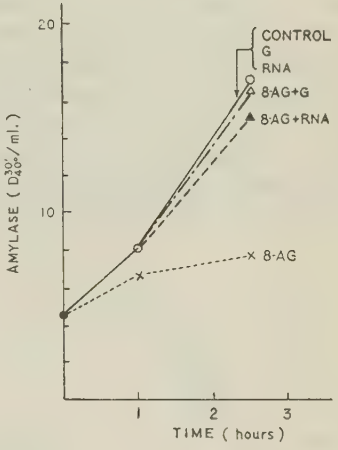

FIG. 3. Inhibition of amylase formation at the early stationary phase by 8-AG and its reversal by guanine or yeast RNA. 5 ml. of ZLE grown culture at the early stationary phase (phase I in Fig. 1) was withdrawn and added to 1.3 ml. of each of the solutions containing the various additions as indicated in the figure. Final concentrations: 8-AG 5×10^{-3} *M*, guanine (G) 1.1×10^{-4} *M*, yeast RNA 4.4×10^{-3} *M* on the basis of tetranucleotide.

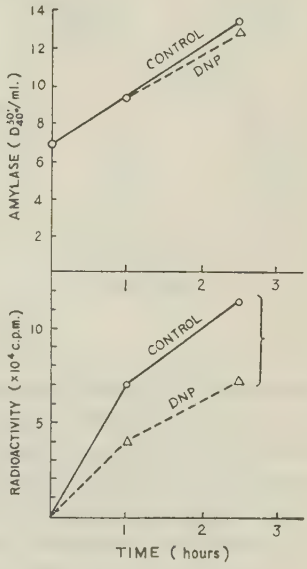

FIG. 4. Effect of 2,4-dinitrophenol(DNP) on the incorporation of P^{32}-inorganic phosphate into the nucleic acid fraction and on the formation of amylase. Cells were grown in LBY (E, 7.5) medium for 18 hours, diluted 6 times with the same fresh medium and further cultivated. After 5 hours, when culture entered stationary phase, 10 ml. of culture was withdrawn, added to 1.3 ml. of P^{32}-phosphate solution with or without DNP as indicated in the figure. Total activity of P^{32} added, 5.3×10^7 c.p.m.; specific activity, 1.6×10^5 c.p.m./μM P. Final concentration of DNP, 0.5×10^{-4} *M*.

pound was added to the stationary phase culture with or without further addition of DNP (0.5 to 1.5×10^{-4} *M*). Aliquots were withdrawn at intervals and both the amount of amylase and that of isotope incorporated into nucleic acid fraction were determined. Thus, the effect of DNP on these processes

were examined simultaneously. The results obtained are presented in Figs. 4 and 5.

After some lag period DNP greatly inhibited the incorporation of both C^{14}-adenylic acid and P^{32}-phosphate into nucleic acid without serious effect on the enzyme formation. Thus, it was concluded that the simultaneous synthesis of nucleic acid from the precursor compounds used in the experiment is not necessary for the amylase formation.

Reversal of 8-AG Inhibition by DNP—From the inhibition experiment using base analogues, several workers maintained that the synthesis of new nucleic acid is essential for the synthesis of enzyme. However, the effective inhibition by analogues does not mean the inhibition of nucleic acid synthesis. Analogues merely replace the normal counterparts and incorporated into the nucleic acid. The incorporation of 8-AG into the RNA of microorganism

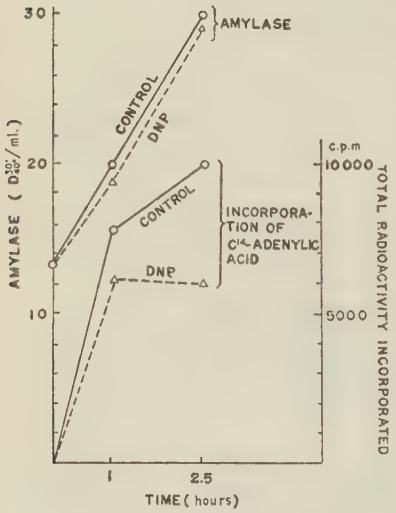

Fig. 5. Effect of 2, 4-dinitrophenol (DNP) on the incorporation of C^{14}-adenylic acid into the nucleic acid fraction and on the formation of amylase. To 5ml. of ZLE-grown culture at stationary phase (Fig. 1), 1.3ml. of C^{14}-adenylic acid solution containing DNP as indicated in the figure (final concentration, $1.5\times10^{-4}M$) was added. Total activity of C^{14}-adenylic acid added, 115,000 c.p.m.; specific activity, 210,000 c. p. m./μM. At the lst and 2.5 th hours, samples were withdrawn and both the amount of amylase and C^{14}-incorporated into nucleic acid fraction were determined.

was reported in several papers (*3*, *11-13*) and it is presumably the case in our experiments. The inhibition by 8-AG of amylase formation is probably due to the formation of abnormal nucleic acid containing 8-AG. If this interpretation is correct, the inhibition by 8-AG may be suppressed by DNP which was shown to inhibit the formation of nucleic acid from the precursor of nucleoside level. The experiment was undertaken to test this possibility.

The cultures at phase I (Fig. 1) were shaken with or without added DNP. After 30 minutes, 8-AG was added to each of these cultures and its effect on the amylase formation was examined. As shown in Fig. 6, the presence of DNP effectively suppressed the inhibitory effect of 8-AG, as expected first. Thus, it was concluded that the inhibition of amylase formation by 8-AG is not due to the inhibition of nucleic acid synthesis, but due to the synthesis of abnormal nucleic acid containing this analogue.

Effect of 8-AG and DNP on the Incorporation of S^{35}-Methionine into Cell Protein—Using S^{35}-methionine an experiment similar to that described in the previous section was carried out to determine whether the inhibition of 8-AG

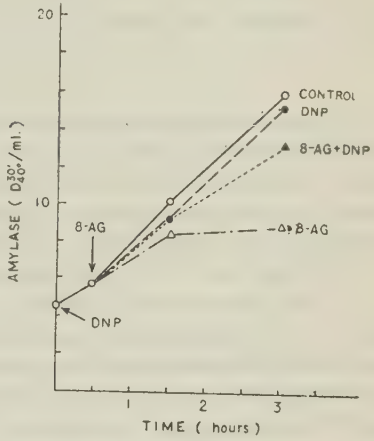

FIG. 6. Inhibition of amylase formation at the early stationary phase by 8-AG and its reversal by DNP. 5 ml. of the ZLE-grown culture was withdrawn at the early stationary phase. DNP was added at time 0 (final concentration 1.0×10^{-4} *M*); 8-AG was added after 30 minutes (final concentration 5.0×10^{-3} *M*). Total volume, 6.3 ml.

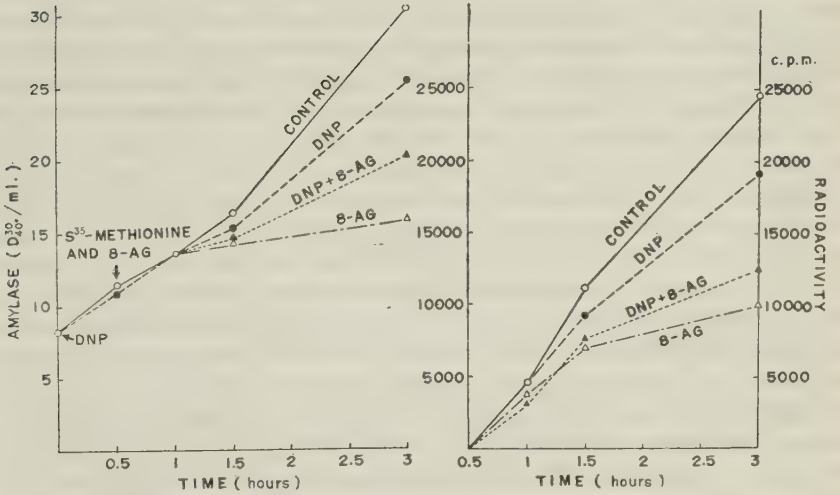

FIG. 7. Inhibition of amylase formation and S^{35}-methionine incorporation into the cellular protein fraction by 8-AG and its reversal by DNP. 8ml. of ZLE-grown culture was withdrawn at the early stationary phase (phase I. in Fig. 1). DNP (final concentration 1.0×10^{-4} *M*) was added at 0 time. 8-AG (5×10^{-3}*M*) and S^{35}-methionine were added after 30 minutes. Total activity of S^{35}-methionine added 370,000 c.p.m.; specific activity, 2460,000 c.p.m./mg.

is specific to the amylase formation or not. S^{35}-methionine was added to the culture and the effect of 8-AG both on the incorporation of S^{35}-methionine into protein and on the amylase formation was tested either in the presence

or in the absence of DNP. As shown in Fig. 7,8-AG inhibited both processes, and this inhibition was suppressed by DNP in the same way as in the case of amylase formation. Therefore, the 8-AG inhibition and its suppression by DNP do not seem to be specific to the amylase formation, but are general in the protein metabolism in this phase.

DISCUSSION

The inhibition of amylase formation in the stationary phase by 8-AG and its reversal by guanine clearly indicated the participation of nucleic acid in the amylase formation. The participation of nucleic acid was also established by the direct isolation of RNA from the cell and the demonstration of its stimulatory effect on the amylase formation (*4*, *5*). However, the mode of participation of RNA is not yet clear, as in the case of other systems.

As to the necessity of continuous RNA synthesis for the enzyme formation, the conclusion does not seem to be made definitely yet. Many workers maintained the necessity of continuous synthesis of RNA for the enzyme formation from the inhibition experiment using base analogues. The argument is not necessarily adequate, as clearly indicated in the experimental results presented in this paper. In our system, enzyme formation was not inhibited by DNP which greatly inhibited the nucleic acid synthesis from the labeled precursor compounds used (P^{32}-phosphate and C^{14}-adenylic acid). The RNA may be synthesized by the condensation of nucleoside-5′-diphosphate as demonstrated in Ochoa's experiment (*14*). The C^{14}-adenylic acid used in the present experiment was the mixture of 2′-and 3′-phosphates. Therefore, the conclusion derived from the present experiment is that the synthesis (or turnover) of RNA from the precursor materials of nucleoside level or from that of smaller compounds may not be necessary for the amylase formation or for the incorporation of S^{35}-methionine into protein. The synthesis of a small amount of RNA from the precursors of nucleotide level or the turnover of RNA to this level was not excluded. In the study of the nature of the active factor (RNA) obtained from the boiled cell extract, it was demonstrated that the mixture of mono- or oligo-nucleotides obtained after the RNA-ase treatment of the factor preparation had still a stimulatory effect on the amylase formation. Probably, the reconstruction of necessary RNA may take place in this case. However, the question, whether such synthesis is obligatory or not, was not answered in this case. The final solution of the problem must await further analysis in the more suitable experimental system, especially in that of lysed cell preparation.

The inhibition of amylase formation by 8-AG was interpreted to be due to the formation of abnormal nucleic acid containing 8-AG. The question, whether the inhibition is caused by the replacement of normal RNA by the abnormal one or by the inhibitory action of abnormal RNA on the coexisting normal one was not answered definitely in the present paper. The isolation of abnormal RNA containing 8-AG and the examination of its effect on the amylase formation in the presence of factor preparation (normal RNA),

especially in the lysozyme-lysed cell system, may be very interesting in this connection. The decrease of biological activity in the abnormal RNA containing 8-AG was reported by Matthews on the plant virus (*13*).

SUMMARY

The relationship between amylase formation and nucleic acid synthesis was studied using 2, 4-dinitrophenol (DNP, $0.5—1.5\times10^{-4}M$) and 8-azaguanine (8-AG, $5\times10^{-3}M$) as inhibitors of nucleic acid metabolism.

Both reagents inhibited the growth of the bacterium and, consequently, the subsequent formation of amylase when added during the logarithmic growth phase. However, DNP showed little inhibitory effect on amylase formation when it was added after the growth ceased, though it could effectively inhibit the incorporation of P^{32}-phosphate and C^{14}-adenylic acid into nucleic acid fraction.

8-AG inhibited amylase formation when added at the stationary phase. This inhibition was reversed by either guanine or yeast RNA. The inhibition was not specific to amylase formation. The general protein metabolism, as measured by the incorporation of S^{35}-methionine into protein, was also inhibited by 8-AG. The inhibition by 8-AG was interpreted not to be due to the inhibition of synthesis of nucleic acid, but due to the formation of abnormal RNA. This interpretation was further supported by the experimental demonstration that DNP effectively eliminated the inhibition by 8-AG.

It was concluded that RNA is necessary for the formation of amylase, but its continuous synthesis (or turnover) from the precursor of nucleoside level may not be required.

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STUDIES ON DENITRIFICATION

III. ENZYMATIC GAS PRODUCTION BY THE REACTION OF NITRITE WITH HYDROXYLAMINE

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In the previous paper we suggested that nitrogen production in denitrification could be due to an anime-nitrite reaction; that is, could result from a series of successive reactions in which a part of the nitrite is reduced to an amine, the remaining nitrite, reacting with this amine, produces gaseous nitrogen (*1*). In our experiments we used dimethyl-*p*- or non-substituted *p*-phenylene diamine as the reaction partner for nitrite, but at that time we could say nothing about the kind of natural amine which is responsible for the reaction in the living cell.

In this paper, based on some similarities in mode of action of dimethyl-*p*-phenylene diamine (DMPPD) and hydroxylamine, we give evidence to support the conception that hydroxylamine may possibly be the natural amine. We describe some experiments indicating that in the absence of lactate, the main gaseous product of the enzymatic reaction of nitrite and hydroxylamine is nitrous oxide, while if lactate is simultaneously present, it is nitrogen.

There are many works indicating that, in addition to nitrogen, nitrous oxide appears as a common gaseous product in denitrification. As yet no one has been able to explain the fact that, in some cases, nitrogen appears as a main product and, in others, that nitrous oxide is produced as a main product. We believe that our experimental results can throw light upon the problem.

Further we describe some experiments on the fractionation of nitrogen-producing enzyme using the DMPPD-nitrite system. These experiments indicate that the nitrogen-producing system has at least two enzyme components.

MATERIAL AND METHOD

The microorganism and procedure of preparation of resting cell suspension were the same as described in the previous paper (*2*).

Acetone powder of denitrifying bacteria was prepared by a common procedure at a low temperature (*cf.* 3.). Cell-free extract was obtained by macerating the acetone powder in *M*/20 phosphate buffer at pH 6.8 and centrifuging the mash at 2500 g. Fractionation of enzyme was carried out by using the DMPPD-nitrite system for the activity test.

In advance we always neutralized hydroxylamine hydrochloride and DMPPD dihydrochloride by NaOH and used immediately.

Sodium hyponitrite was prepared by the method described by Antonia Medina

and D.J.D. Nicholas (*4*). Before use, the silver salt of hyponitrous acid was converted into sodium salt by treating it with NaCl and the concentration of hyponitrite in the solution was colorimetrically determined as nitrite after hyponitrite was oxidized to nitrite by the addition of iodine.

The method of estimation of gas evolution was the same as described in the previous paper (*2*), namely, estimation was made under the condition of strict exclusion of oxygen from the gas phase at pH 6.8–7.4 at 30°, using Warburg apparatus. As will be shown later, we certified by means of Warburg's indirect method for the estimation of two kinds of gases of different solubility in water, that gas evolved from NH_2OH-$NaNO_2$ reaction must be calculated as nitrous oxide. The gas produced on decomposition of hyponitrite was also calculated as nitrous oxide. In every other case we used k_{N_2} as the flask constant.

RESULTS

Effects of Formamide, Asparagine, Hydrazine and Ammonium Sulfate—Anyone of these substances were added along with nitrite to resting cell suspension in the absence or presence of lactate. In the absence of lactate formamide and asparagine can act as hydrogen donors in the same way as lactate and the amounts of gas evolved corresponded to nitrite-nitrogen of the reaction system, but hydrazine and ammonium sulfate do not enhance the gas evolution appreciably. In the presence of lactate, all these substances had little effect on either the rate of reaction or the volume of nitrogen produced.

Effect of Hydroxylamine—In manner of action, hydroxylamine appeared to be thoroughly different from the above mentioned substances. The rate of production and the volume of gas produced when $M/100$ NH_2OH was added to nitrite in the presence of lactate, were found to be 1.7 times as much as that produced under the condition without the addition of NH_2OH (Fig. 1).

Further, hydroxylamine itself was shown to react with nitrite in the absence of lactate and the volume of gas produced in this system was about twice (2.1–2.2 times) the volume which can be produced from nitrite and lactate, as shown in Fig. 2. Placing acidic permanganate in the center well of Warburg vessel to absorb nitric oxide, we were able to find that this gas did not contain nitric oxide. An anaerobic breakdown of NH_2OH alone into the gaseous product by the bacteria could not be detected.

Michaelis Constants in the Hydroxylamine-nitrite System—Apparent Michaelis constants were determined by using cell-free extract. The Michaelis constant of the denitrifying enzyme for nitrite at pH 6.8, 30° was estimated to be about $10^{-3.8}$ mole per liter when the concentrations of hydroxylamine were fixed at $M/10$ and $M/100$. The Michaelis constant for NH_2OH was about $10^{-1.2}$ mole per liter when the concentration of nitrite was fixed at $M/448$.

When concentration of nitrite was above $M/10$, a remarkable non-enzymatic gas production was observed at pH 6.8. even if the concentration of hydroxylamine was $M/20$. As for NH_2OH, when concentration of nitrite was lower than $M/224$, this non-enzymatic gas evolution was not observed even if the concentration of NH_2OH was as high as $M/2$. In our experimental conditions, therefore, the non-enzymatic reaction could be neglected entirely.

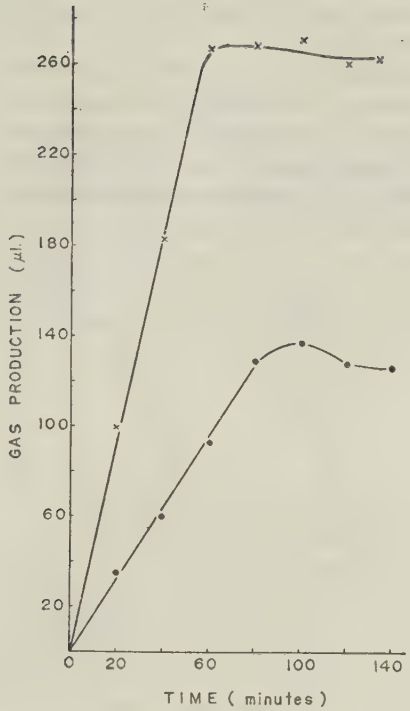

FIG. 1. The effect of hydroxylamine in the presence of lactate. Side arm (two side arm's vessel); $NaNO_2$ $M/22.4$ 0.3 ml., NH_2OH $M/10$ 0.3 ml. Main compartment; cell suspension 0.5 ml. phosphate buffer $M/2$ pH 6.6 0.3 ml., sodium lactate $M/2$ 0.3 ml. Center well; 20 per cent KOH (with filter paper) 0.2 ml. Total volume; 3.2 ml. Gas phase: nitrogen.

—●— Nitrite was tipped in to cell suspension.

—×— Hydroxylamine and nitrite were tipped in to cell suspension.

—△— Hydroxylamine was tipped in to cell suspension.

—▽— Hydroxylamine and nitrite were tipped in to boiled cell suspension (at 100° for 10 minutes)

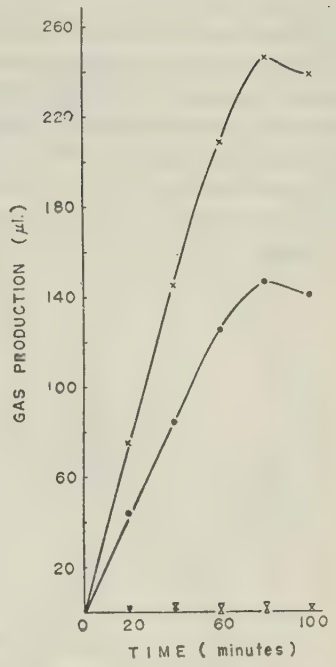

FIG. 2. The reaction of nitrite with hydroxylamine. Side arm; hydroxylamine $M/2$ 0.3 ml. Main compartment; cell suspension 0.8 ml., phosphate buffer $M/2$ pH 6.8 0.3 ml. sodium nitrite $M/22.4$ 0.3 ml., sodium lactate $M/2$ 0.3 ml. Center well; 20 per cent KOH (with filter paper) 0.2 ml. Total volume; 3.2 ml. Gas phase; nitrogen.

● - Nitrite was tipped in to cell suspension in the presence of sodium lactate (in this case nitrite was put in side arm).

—×— Hydroxylamine was tipped in to nitrite and cell suspension in the absence of sodium lactate.

Inhibitions in the Hydroxylamine-Nitrite System—In these experiments the concentration of nitrite was $M/224$ or $M/448$, and hydroxylamine was used in

high concentrations, usually in $M/20$, becaus the Michaelis constant for hydroxylamine was very high as mentioned above.

The activity of denitrifying enzyme was wholly lost by heating at 100° for 10 minutes and inhibited 80–90 per cent by 10^{-3} M KCN. By *p*-chloromercuribenzoate, which was shown to be a strong inhibitor in the system of nitrite with lactate or with DMPPD (*1*, *2*), the enzyme was inhibited only 15-20 per cent at most, when the concentration of hydroxylamine used was $M/20$. Accounting for the possibility that *p*-chloromercuribenzoate com-

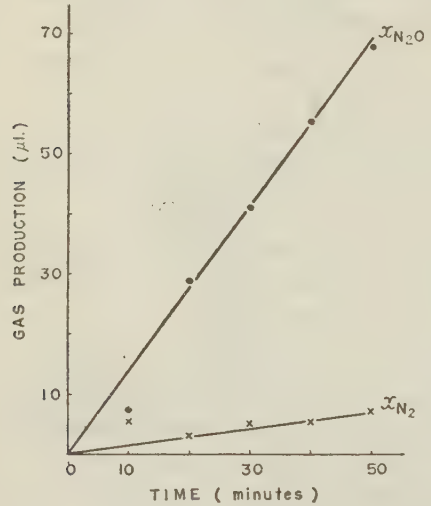

Fig. 3a. Gas analysis with NH_2OH-$NaNO_2$ system by Warburg's "indirect method". Side arm; resting cell suspension 0.3 ml. Main compartment; phosphate buffer $M/2$ pH 6.8, $NaNO_2$ $M/22.4$, NH_2OH $M/2$ (The end concentrations of these substances are 1/10 each). As the presence of alkali in the center well had no influence on the gas production, it was avoided. Gas phase; nitrogen. In the first flask total volume was 1 ml. and in the second, 8 ml.

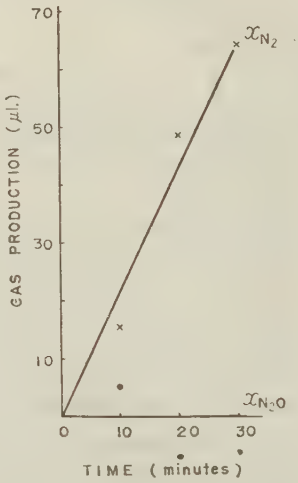

Fig. 3b. Gas analysis with NH_2OH-$NaNO_2$ system in the presence of lactate by Warburg's "indirect method". Side arm; resting cell suspension 0.3 ml. Main compartment; phosphate buffer $M/2$ pH 7.1, $NaNO_2$ $M/22.4$, NH_2OH $M/10$, Na lactate $M/2$ (The end concentrations of these substances are 1/10 each). Center well; 20 per cent KOH 0.2 ml. In the first flask total volume was 1.2 ml. and in the second, 5.2 ml. Gas phase; nitrogen.

bines with NH_2OH, we used a lower concentration of NH_2OH, *i.e.*, $M/100$, in which case the inhibition increased up to 45 per cent. We also found that the enzyme is made inactive by high concentrations of NH_2OH. For instance, $M/20$ NH_2OH inactivated the enzyme within 20 minutes and the presence of a small amount of nitrite protected it from inactivation.

Gas Analyses by Warburg's "Indirect Method"—After considering the probable mechanism of the reaction of nitrite and hydroxylamine (see

Discussion), it is most likely that, in this system nitrous oxide is produced, even should nitrogen also be produced simultaneously. We therefore set to work to obtain information on the composition of gas produced in the NH_2OH-$NaNO_2$ system by using Warburg's "indirect method". The experimental facts can best be explained by the conclusion that, in the absence of lactate, the evolved gas consists of nitrous oxide almost exclusively and that in the presence of lactate, the whole gas (including the 70 per cent excess volume caused by addition of NH_2OH) is nitrogen alone (Fig. 3a, 3b). Because of the inevitable inaccuracy of estimation by the indirect method, more precise details of the composition of evolved gas must be obtained by gas analysis experiments. These we are now undertaking.

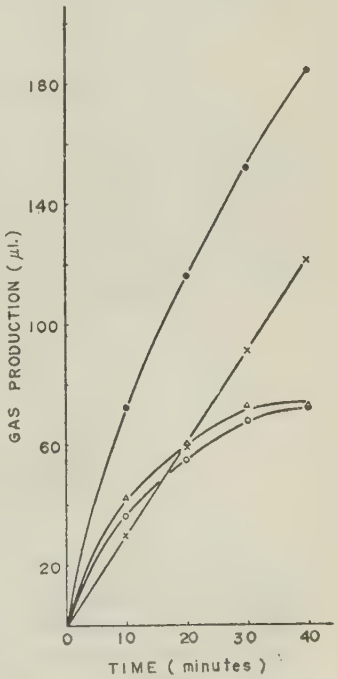

FIG. 4. Gas production from hyponitrite and nitrite. Side arm; resting cell suspension 0.3ml. in the first side arm. sodium hyponitrite *M*/40 0.3 ml. in the second side arm. Main compartment; phosphate buffer *M*/2 pH 7.1 0.3 ml., Na lactate *M*/2 0.3 ml., Na nitrite *M*/22.4 0.3 ml. Center well; 20 per cent KOH 0.2 ml. (with filter paper). Total volume; 3.2 ml. Gas phase; nitrogen.

—○— spontaneous decomposition of hyponitrite.

—△— decomposition of hyponitrite in the presence of resting cell suspension.

—×— nitrogen evolution by resting cell suspension from nitrite.

—●— gas production by cell suspension from nitrite+hyponitrite.

Experiments on Hyponitrite—As there was a possibility that hyponitrite could be produced in the NH_2OH-$NaNO_2$ reaction, we tried to use sodium hyponitrite as a hydrogen acceptor or as a substrate to be decomposed enzymatically. We could unequivocally demonstrate the fact that hyponitrite can not be utilized as shown in Fig. 4, a result the same as reported by M. B. Allen and C. B. van Niel (*5*) and A. Kluyver and W. Verhoeven (*6*). Since hyponitrite is decomposed spontaneously at quite a high rate in neutral solutions, it might be possible for hyponitrite to be an intermediate stage in the reaction process. This consideration is hardly probable, however, because spontaneous decomposition takes place independently of the presence of lactate and bacteria.

Experiments with DMPPD-Nitrite System—In measuring gas production in using DMPPD and nitrite, a short lag was always observed, so that it is necessary to determine the reaction rates from the straight line part of gas output-time curves.

In the above mentioned conditions, Michaelis constant of the enzyme for nitrite at pH 6.8 and 30° was found to be about $10^{-3.4}$ mole per liter and the Michaelis constant for DMPPD was $10^{-1.5}$ mole per liter. These value were very close to those in the NH_2OH-nitrite system. The net rate of gas production in the saturated conditions is several times greater in the DMPPD-nitrite system than that in the NH_2OH-nitrite system, if the measure-

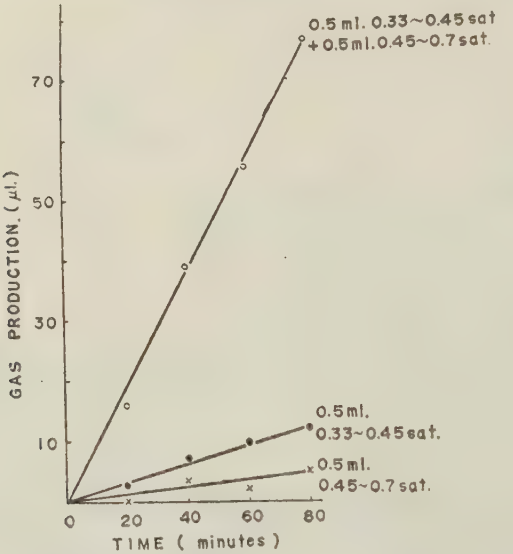

Fig. 5. Gas production with the components obtained by ammonium sulfate fractionation. Side arm; sodium nitrite *M*/5 0.3 ml. Main compartment; enzyme solution, phosphate buffer *M*/2 pH 7.1 0.3 ml., DMPPD *M*/2 0.3 ml. Center well; 20 per cent KOH (with filter paper) 0.2 ml. Total volume; 3.2 ml. Gas phase; nitrogen. Each addition of the components is noted on the right hand side.

ment were done with an equivalent amount of cell-free enzyme in both systems.

Some attempts to purify the denitrifying enzyme were undertaken in the following manner. Most of the activity was found to be contained in the supernatant obtained by centrifuging cell-free extract at 20,000 g. The fractionation of this supernatant was carried out by adding solid ammonium sulfate at pH 6.8. By saturation of 0.33–0.45 and of 0.45–0.7 we obtained two fractions. Both fractions were dialyzed in cellophane bags against distilled water for 18 hours. Neither fraction alone was shown to be capable of producing gas when substrates are added; but with mixing both fractions, a

remarkable gas production could be realized (Fig. 5).

DISCUSSION

Let us consider the reaction of nitrite and hydroxylamine under the action of the bacterial enzyme. In the absence of the lactate-lactic enzyme system, the amount of gas produced corresponds to twice the amount of gas calculated from the nitrite-nitrogen alone, and in the presence of the lactate-lactic enzyme system to 1.7 times. We can consider no other possibility than that these gases consist of either N_2 or N_2O or a mixture of both. In either case, it is a certainty that NH_2OH-N reacts with $NaNO_2$-N in converting the N of the latter into one mole of a gaseous product. In the absence of hydrogen-donating systems other than hydroxylamine itself, the interpretation of the reaction should be given by

$$HNO_2 + NH_2OH = N_2O + 2H_2O$$

Th precise mechanism of this enzymic reaction is unknown at present.

In the presence of a hydrogen-donating system, such as lactate-lactic enzyme, we can employ a scheme analogous to the scheme used for the reaction between nitrite and DMPPD (*1*), in which we at first considered nitrite to be reduced to nitric oxide. Our next consideration is that nitric oxide reacts with hydroxylamine to produce molecular nitrogen where one atom equivalent of hydrogen is consumed as shown in the following:

$$HNO_2 \xrightarrow{+H} NO + H_2O$$

$$NO + NH_2OH \xrightarrow{+H} N_2 + 2H_2O$$

$$HNO_2 + NH_2OH \xrightarrow{+2H} N_2 + 3H_2O$$

Summarizing our experimental facts, we offer the following scheme as the probable mechanism of denitrification:

$$HNO_2 \xrightarrow{+4H} NH_2OH \xrightarrow{+HNO_2} N_2O$$

$$NH_2OH \xrightarrow[+2H\ (e.g.\ \text{from lactate})]{+HNO_2} N_2$$

At any rate, hydroxylamine represents a substance which can satisfy the necessary condition for the proper amine postulated beforehand, although it is premature to assert at present that hydroxylamine is the true intermediate in the denitrification. Of course it may not be denied that in other species of denitrifying bacteria some different mechanism of denitrification could be responsible.

Referring to this, it is interesting to note that at least two components may possibly participate in nitrogen production in the DMPPD-nitrite system.

SUMMARY

1. When hydroxylamine is added to nitrite in the presence of lactate by using resting cell suspension, both production rate and volume of nitrogen are increased remarkably.

2. Hydroxylamine alone reacts with nitrite and forms a gaseous product, probably nitrous oxide, two nitrogen atoms of which are supposed to originate, one from hydroxylamine and the other from nitrite. This production of gas is inhibited by 10^{-3} *M* KCN or by a high concentration of hydroxylamine, and the enzyme is destroyed by heating at 100° for 10 minutes. Accordingly there is no other way than to consider that the reaction is enzymatic.

3. Hyponitrite has no effect on production of nitrogen from nitrite; it behaves as an indifferent substance.

4. For NH_2OH-$NaNO_2$ system, the Michaelis constants were estimated using cell-free extract. The values are $10^{-1.2}$ mole per liter for NH_2OH and $10^{-3.8}$ mole per liter for $NaNO_2$. Experiments with DMPPD-$NaNO_2$ system indicate that the Michaelis constants are $10^{-1.5}$ mole per liter for DMPPD and $10^{-3.4}$ mole per liter for $NaNO_2$

5. It was found that in the DMPPD-$NaNO_2$ system, there is participation by two enzyme components which are separated by a fractionation of the cell-free supernatant obtained by centrifuging at 20,000×*g*. using ammonium sulfate.

6. The essential reaction stages in the denitrifying process can be presented in the simple formula

$$NHO_2 \xrightarrow{+4H} NH_2OH \xrightarrow{+HNO_2} N_2O$$
$$\Big\downarrow {\scriptstyle +HNO_2,\ +2H}$$
$$N_2$$

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